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Title: Clinical application of biomarkers in colon cancer : studies on apoptosis, proliferation and the immune system

Issue Date: 2014-09-17

Clinical application of biomarkers in colon cancer

studies on apoptosis,
proliferation and the
immune system

Eliane Zeestraten

Clinical application of biomarkers in colon cancer
studies on apoptosis, proliferation and the immune system
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Colophon

Clinical application of biomarkers in colon cancer, studies on apoptosis, proliferation and the immune system

Thesis, Leiden University, The Netherlands, 2014

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ISBN/EAN 978-94-6108-727-0

NUR 870

Cover art: Texcetera, Den Haag

Layout: Texcetera, Den Haag

Printed by: Gildeprint, Enschede

The financial support provided by Boehringer Ingelheim, Covidien, Roche, Novartis Oncology, Erbe, Chipsoft, ABN Amro, ISA Pharmaceuticals, Olympus Nederland B.V. and Convatec for the printing of this thesis is gratefully acknowledged.

Clinical application of biomarkers in colon cancer

studies on apoptosis, proliferation and the immune system

PROEFSCHRIFT

Ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof. mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdediging op 17 september 2014
Klokke 15:00

door

Eliane Cornelia Maria Zeestraten

geboren te Heerlen in 1983

Promotiecommissie

Promotor: Prof. dr. C.J.H. van de Velde

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CHAPTER 1

General introduction and thesis outline

Colon cancer is a major contributor to cancer-related mortality worldwide. Death from colon cancer occurs in the majority of the cases from widespread metastatic disease. Surgery alone can cure a large proportion of colon cancer patients presenting with non-metastasized disease (1;2). Without any adjuvant treatment, up to fifty percent of these patients will not develop disease recurrence or metastasis following surgery (2-4).

Despite optimal surgical therapy, between 30-50% of initial stage II or III colon cancer patients will still suffer from metastatic disease (2-4). This risk can significantly be reduced by applying postoperative or adjuvant chemotherapy. Studies have however shown that only a selected proportion of the stage II and III patients will actually benefit from adjuvant treatment because not all patients within this cohort will develop a distant metastasis and because of low response rates to therapy, in the case of stage II disease this might even only be 15% of the patients (5;6). The decision to offer adjuvant therapy, especially for stage II disease, should therefore be balanced against the possible risks of treatment-related toxicity (6). This makes it essential for the clinician to be able to precisely identify the high risk patient cohort.

Nowadays, treatment allocation is based on tumor staging using only the TNM (Tissue Node Metastasis) criteria developed by the IUCC (Union Internationale Contre le Cancer) and the AJCC (American Joint Committee on Cancer) that are applied worldwide (7). The application of the TNM criteria, based on tumor morphological characteristics, falls short in the identification of the high risk patient population. Prognostic biomarkers that provide additional information on patient outcome might improve staging criteria. Therefore, increasing attention is now being directed towards the discovery of prognostic biomarkers to identify high risk colon cancer patients. Several methods have been used, but two main approaches can be distinguished.

A genomic approach that involves the simultaneous examination of a large number of genes using complex platforms, including multigene-based mutation assays and gene expression microarray technologies. The power of this approach lies within its capacity to tackle tumor heterogeneity with the goal to develop a unique genetic signature to provide the opportunity to match therapy to the characteristics of the individual patient's tumor (8). So far, despite numerous studies reporting on different prognostic gene sets, only two genomic profiles have been validated in independent patient cohorts. These are the ColoPrint and the 12-gene colon cancer recurrence score (9-11). The colon cancer recurrence score has been offered by the Genomic Health company since January 2010 for clinical use as the Oncotype DX® Colon Cancer Assay and is now available to support treatment planning for stage II and stage III colon cancer patients (12). A point of discussion in the use of these signatures is the lack of strong biological basis (13). As only a small number of the genes from the different gene expression signatures actually overlap and only a weak interaction was observed between the gene expression signatures and tumor stage (13-16). This lack of interaction between gene expression signatures and tumor stage has been found in many studies, for example, in a large validation

study of the colon recurrence score developed by the NSABP (National Surgical Adjuvant Breast and Bowel Project) but also in the second validation study of the ColoPrint (9;11). Furthermore, in this last study the results for risk recurrence, based on the ColoPrint and those based on several clinical tumor biology-based factors such as, T-stage and tumor grade were discordant in 50% of the patients indicating a lack of interaction of the signature with actual tumor biology (9). Probably in the future of the clinical decision making process besides gene expression signature, other clinico-pathology-based markers may be used to achieve the most accurate risk assessment.

The second approach is a more tumor biology-directed approach, with a focus on biological determinants of the tumor's metastatic potential. The biological hallmarks of cancer are six biological capabilities a cell acquires during the process of tumorigenesis (17). They include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. Recently, two 'emerging hallmarks', features that tumors gain during their development, were added: reprogramming of energy metabolism and evading immune destruction (17;18). In this thesis we focus especially on three hallmarks of tumor development in colon cancer in order to identify biomarkers of disease recurrence and to develop new treatment strategies based on this biological approach. In part 1 the focus is on markers of apoptosis and proliferation, and in part 2 on tumor-immune interactions.

AIMS AND OUTLINE

Part 1 Biomarkers of apoptosis and proliferation

A key factor in colonic tissue homeostasis is the balance that exists between cellular apoptosis, or programmed cell death, and the level of cellular proliferation (19-21). Several hallmarks of cancer development such as sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality together determine a balance between cell proliferation and cell death. The outcome of this balance determines tumor growth (17). Therefore, biomarkers representing these pathways might harbor the potential to identify high risk stage II colon cancer patients. Previously, we have been able to determine a prognostic value of the level of apoptosis in rectal cancer patients (22;23). In colon cancer this value has been debated (20;24-27). The aim of the first part of this thesis was to determine the clinical prognostic value of apoptosis and proliferation in colon cancer patients and to develop biomarkers with the potential to be used in a clinical setting to identify high risk stage II colon cancer patients. Chapter 2 provides a review of the current literature of biomarkers of apoptosis in colorectal cancer in order to identify any biomarker that has already been proven to be of clinical prognostic value. In chapter 3 a combined analysis of biomarkers of proliferation and apoptosis, determined with immunohistochemistry in a large cohort of colon

cancer patients, is evaluated for its prognostic quality. Chapter 4 describes the development and identification of a new prognostic, biochemically determined, biomarker based on tumor cell proliferation in stage II colon cancer patients: the CDK1 SA (Cyclin Dependent Kinase 1 Specific Activity). Finally in chapter 5 a biomarker combination consisting of two biochemical assays reflecting the level of apoptosis and proliferation in colon cancer tissue is studied for the clinical identification of high risk stage II colon cancer patients.

Part 2 Tumor immune interactions

In part 2 the focus is on one of the emerging hallmarks in cancer biology: evading immune destruction (18). Studying tumor immune interactions not only provides us with new biomarkers of tumor aggressiveness, it will also open a door to new cancer treatment strategies. Besides focusing on chemotherapy as adjuvant treatment strategy for high risk stage II patients, exploiting possibilities of activating the patient's immune system as a therapeutic modality is becoming an emerging modality as well. Chapter 6 gives an overview of the colorectal cancer vaccines that have been studied in clinical trials until now including the first studies with the p53-SLP vaccine which is used in a clinical phase I/II trial as described in chapter 7. Although the results of these studies are hopeful, as we were capable of eliciting vaccine-specific T-helper responses, optimal patient selection in advance may make real clinical breakthroughs possible. Therefore, in chapter 8 we focus on the determination of key factors in tumor-immune interactions such as tumor expression of HLA class I and local presence of Foxp3- and CD8-positive cells and their prognostic value. In chapter 9 we combine this knowledge into a clinical prognostic tumor-immune phenotype that might eventually aid us in the identification of high risk stage II colon cancer patients and select those that might benefit from adjuvant vaccination or chemotherapy treatment strategies.

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PART
ONE

**BIOMARKERS
OF
APOPTOSIS
AND
PROLIFERATION**

CHAPTER 2

The prognostic value of the apoptosis pathway in colorectal cancer: A review of the literature on biomarkers identified by immunohistochemistry

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G. Liefers, C. van de Velde, P. Kuppen.

Biomarkers in cancer. 2013: 5 13-29

ABSTRACT

Research towards biomarkers that predict patient outcome in colorectal cancer (CRC) is rapidly expanding. However, none of these biomarkers have been recommended by the American Association of Clinical Oncology or the European Group on Tumor Markers. Current staging criteria result in substantial under- and over treatment of CRC patients. Evasion of apoptosis, a characteristic feature of tumorigenesis, is known to correlate with patient outcome. We reviewed the literature on immunohistochemistry-based studies between 1998 and 2011 describing biomarkers in this pathway in CRC and identifying 26 markers. Most frequently described were p53, Bcl-2, survivin and the Fas and TRAILR1 receptors and their ligands. None of the studies reviewed provided sufficient evidence for implementing a single marker into current clinical practice. This is likely due to the complex biology of this pathway. We suggest focusing on the combination of key markers within the apoptosis pathway that together represent an 'apoptotic tumor profile', which better reflects the status of this pathway in a tumor.

INTRODUCTION

Colorectal cancer (CRC) is currently one of the major contributors to cancer-related deaths worldwide (1;2). The amount of data emerging from studies aimed at optimizing the diagnostic process and treatment of this disease is rapidly increasing. This makes the process of tumor development in CRC one of the most thoroughly studied and best characterized models of tumorigenesis. By emphasizing the need of early detection and development of new and improved treatment regimens, an increased understanding of the disease did led to decreased mortality rates of nearly 5 percent over the last decade (3-10). However, CRC -related morbidity and mortality affects approximately 800,000 individuals each year worldwide (2). The survival of CRC patients largely depends on disease stage at the time of diagnosis and varies widely between the stages. In clinical practice, however, treatment allocation and outcome prediction is still solely based on the International Union Against Cancer (UICC) Tissue Node Metastasis (TNM) classification (11). Addition of several pathology -based tumor characteristics is currently used to identify high-risk stage II patients that may benefit from adjuvant chemotherapy. These include perforation of the bowel wall at presentation, tumor invasion at the T4 level, venous tumor invasion, lymph node yield less than 10, and poor or no differentiation of the tumor cells (12). There is substantial evidence that even with the addition of these risk factors of poor outcome, TNM classification falls short in daily practice and may cause over- or, even worse, under-treatment of patients (11;13-15;15-18).

In an attempt to improve treatment outcomes for CRC patients, both the American Society of Clinical Oncology's Tumor Markers Expert Panel (ASCO TEMP-2006) and its European counterpart; The European Group on Tumor Markers (EGTM-2007) have reviewed the available literature to determine the clinical applicability of a number of widely studied biomarkers (19-21). Their conclusions were clear and consistent: despite the overwhelming amount of literature, no biomarkers have been recommended for clinical use. Therefore, to improve current staging criteria, new biomarkers must be identified and validated for clinical use. Pepe *et al.* have developed a five-step program that can be used for the development of new biomarkers (22). The first step is biomarker discovery in a preclinical, exploratory setting. Subsequently, the clinical value of these biomarkers must be determined and verified in a large retrospective study. Those results then need to be the validated and eventually confirmed by a prospective randomized controlled trial. It is not until these steps are completed successfully that biomarkers are ready for introduction into clinical practice. The first step which involves identifying or discovering new biomarkers, can be accomplished by studying the process of tumorigenesis and its related pathways. Cancer cells harbor at least six features that distinguish them from normal cells, one of which is the characteristic ability to evade programmed cell death or apoptosis (23). In normal tissues apoptosis plays a pivotal role in the maintenance of tissue homeostasis and the development of the immune

system (24;25). Disturbance of this process in tumor cells results in the impaired removal of mutated cells and contributes to tumor progression. In addition, evasion of apoptosis enables malignant cells to escape from tumor immune surveillance and to acquire resistance to cancer therapy. In previous retrospective studies, the status of the apoptotic pathway in a tumor was shown to be of prognostic value in colorectal cancer patients (26-37). Therefore, we focused on this pathway in our search for new potential prognostic biomarkers in colorectal cancer. In this review, we provide an overview of studies designed to determine the prognostic value of biomarkers within the apoptotic pathway in colorectal cancer. Furthermore, we will discuss some of the difficulties and controversies that can arise when studying this tightly regulated and complex process. The goal is to identify key biomarkers in the apoptotic pathway that may be used clinically to determine cancer prognosis. We first discuss the route of apoptosis to identify the key proteins in this process and then link this information to studies that examined the prognostic value of these proteins in colorectal cancer. Since immunohistochemistry (IHC) is still the most widely applied and available technique in pathology to determine the expression status of tumor-associated proteins and to study the clinical prognostic relevance of biomarkers, we limited our search to IHC studies.

DATA COLLECTION AND ANALYSIS

In order to review the literature on prognostic biomarkers related to the pathway of apoptosis, determined using IHC in CRC patients, we performed a search of the Pubmed, Embase and Web of Science databases. We used broad search terms, as recommended in the Stroup guidelines (38), to identify publications of interest published between January 1998 and June 2011. Key search terms included *colorectal cancer*, *biomarker*, *apoptosis*, *prognosis*, and *immunohistochemistry*. The following search strategy (simplified) shows how some of these terms were combined in our Web of Science Search; “*TS=((colorectal or colon or colonic or rectal or rectum) SAME (neoplasm or cancer or tumor or carcinoma)) AND TS=((prognostic or tumor or cancer or neoplasm or biological or intracellular or signaling or intracellular signaling) SAME (marker or protein or peptide)) AND TS=((prognosis or prognostic or morbidity or mortality or recurrence or relapse or (disease SAME progression))) AND TS=(immunohistochemistry or immunolabeling or immunocyto histochemistry)*”. After amalgamating the results from the three medical databases and discarding the duplicates, this strategy yielded a total of 2923 unique citations. To extract papers for review, we screened the results for title and abstract. We used the following criteria to determine whether a study was considered eligible for the review:

- a) The study contained data on a marker directly involved in the pathway of apoptosis;
- b) The study was performed in primary tumors from CRC patients;
- c) The study was performed using IHC;

- d) The study contained an analysis of the relationship between expression of the marker and clinical outcome. We selected only studies that used logistic regression or survival curve -based statistical analysis methods to evaluate the impact of a marker;
- e) A full publication in English with details of the method used was available.

RESULTS

Overall, we were able to identify 26 potentially prognostic biomarkers that are directly involved in the apoptotic pathway, which will be discussed in detail below (figure 1). These markers were all studied using IHC in the 124 eligible publications that remained after applying our selection criteria from the total of 2923 publications. Expression patterns of these apoptotic (bio)markers were related to patient outcome using logistic regression or survival curve -based analysis methods. Most of the papers, over 800, were excluded because they described the expression of markers related to the pathway of apoptosis in other types of cancers than colorectal cancer, despite the fact that our search terms included colorectal cancer as a major search term. Over 900 citations were excluded because they did not describe the marker in primary colorectal cancer lesions but rather in metastatic lesions. Table 1 provides an overview of our selection criteria and the corresponding number of citations that were excluded based on these criteria.

The general pathway of apoptosis is illustrated in figure 1 and includes the markers that discussed in this review. Although this figure represents a simplified version of the pathway, it shows that the process of apoptosis is highly regulated at multiple levels. Based on the stimuli presented, two pathways initiating the apoptotic process can be identified (39). The extrinsic pathway is triggered by external death signals that cause the formation of intracellular signaling complexes at the death receptors. This type of apoptosis is usually activated in immune responses (40). The second pathway, known as the intrinsic pathway, is activated by many different stimuli, including growth factor deprivation and DNA damage, caused by factors such as UV or gamma-irradiation or by chemotherapeutic agents. Exposure of cells to these stimuli initiates a set of intracellular death signals mediated by the p53 protein that activates the apoptotic process. Mitochondria play an important role in the intrinsic pathway with a major regulatory role for the Bcl-2 family members. Although already intimately connected via caspase-8 and Bid, both pathways converge at the level of the caspase -cascade that eventually leads up to the proteolytic activation of the executioner members such as caspase-3 (41). The function of the caspase proteins is again highly regulated by a group of so-called inhibitor of apoptosis proteins (IAPs) (42). In general, executioner caspases will cleave several substrates, thus acting as a cellular disassembly machine. Cleavage of these substrates is eventually responsible for the morphological features that hallmark apoptotic cell death including membrane blebbing, cell shrinkage and chromatin condensation (26;41).

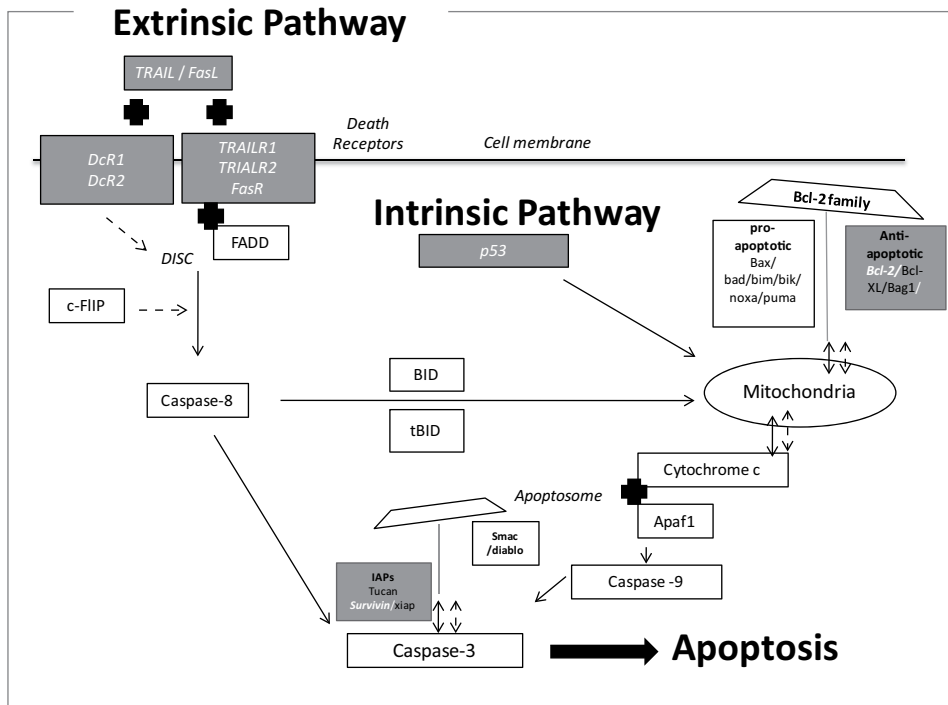


Figure 1

The pathway of apoptosis. A simplified schematic view of the intrinsic and extrinsic pathway of apoptosis and their regulators as described in this review. A regular arrow indicates a positive (stimulating or activating) effect of a regulator on a component in the pathway it points out to. A plus sign indicates that there is an interaction between two components. The combination of these two components exerts a stimulating effect on the progression of the apoptotic cascade. A dotted arrow indicates an inhibitory effect of a regulator on another member or on an activating step within the apoptotic pathway. The markers in italic white in the grey boxes were found to have the most clinical prognostic significance in colorectal cancer patients, based on the studies that resulted from our search of the literature. These markers, their function, and whether they are of true prognostic value are discussed in detail in this review.

To discuss the results of our literature review in an orderly fashion, markers are grouped and discussed based on their location in the pathway as described in figure 1, starting with the extrinsic pathway of apoptosis and ending with the IAPs. In addition, table 2 provides a general overview of the number of studies identified that describe the prognostic value of a particular marker, grouped by their function and location in the pathway of apoptosis in the order at which they will be discussed in this review.

The Death Receptor Family and the extrinsic pathway of apoptosis

The most studied signaling pathway in apoptosis is the extrinsic pathway, activation of which is primarily facilitated by the death receptors (DRs). Based on our search we were able to identify 8 individual biomarkers in this part of the apoptotic

Table 1

Selection of relevant studies on clinical prognosis of apoptosis-related markers

Exclusion criteria and the number of citations that were excluded based on these criteria
(Total number of citations reviewed n=2923)

Exclusion Criteria	Numer of citations excluded
A. The study describes data on a marker not directly involved in apoptosis	642
B. The study was not performed in primary colorectal cancer patients	985
C. The study was not performed using immunohistochemistry	707
D. The study did not contain validated outcome results	315
E. An English full text version was not available	150
Total number of citations excluded based on these exclusion criteria	2799
Total number of citations included (2923-2799)	124

Table 1 provides an overview of the exclusion criteria that were used to select the most relevant citations. The criteria were applied to the 2923 citations retrieved in our search of literature in three major online medical databases. The material and methods section provides further background on the postulation of these criteria and outline of our literature search.

The number of citations excluded from further analyses based on each criterion is listed. Based on the criteria A, B, C, D, and E, 2799 citations were excluded from the selection. Therefore, 124 citations remained for in depth review of the prognostic value of the markers studied.

pathway of which the prognostic relevance was studied in CRC patients. These markers included: Fas receptor (FasR), TRIAL1, TRAILR2, TRAILR3, TRAILR4, FasL, TRAIL, and c-Flip (table 2). A unique feature of the extrinsic pathway is that DRs can induce apoptosis independently of the p53 tumor suppressor gene. DRs are members of the tumor necrosis factor receptor superfamily, of which eight family members have been characterized (43;44). The most common receptors are FasR (CD95, DR2) with its ligand FasL and TRAILR1 and -R2 (DR4 and DR5) with their ligand TRAIL (Tumor necrosis factor (TNF)-Related Apoptosis-Inducing Ligand). All DRs contain cysteine-rich extracellular domains that allow them to recognize their ligands with great specificity. They also harbor intracellular sub-domains better known as the Death Domains (DDs) (45). DDs allow them to interact with adapter molecules such as FADD (Fas-associated Death Domain) (46). Signal transduction within the extrinsic pathway starts with the binding of the ligands to the DRs, followed by the formation of multi-protein signaling complexes called death inducing signaling complexes (DISCs) at the intracellular domains of the DRs (47;48). The DISC complex auto-activates pro-caspase 8 through interaction with a FADD protein. Activated caspase-8 will eventually activate the effector caspase-3 by proteolytic cleavage (48-51). Downregulation of any of the DRs or downstream apoptotic proteins might cause severe limitations in the induction of apoptosis through the extrinsic pathway. There are two other mechanisms involved in the regulation of the extrinsic signaling pathway. First, TRAIL can also bind to two decoy receptors (DcR) in addition to the DRs, including DcR1 and DcR2 (also known as TRAILR3 and TRAILR4, respectively). However, neither decoy receptors can transduce an apoptosis-stimulating signal upon TRAIL binding. The sensitivity of a cell to TRAIL-

Table 2

Overview of markers of the apoptosis pathway

Overview of the markers reviewed, the total number of citations including the references that described the prognostic relevance of these markers

Marker	Function in the pathway	Number of studies	References
Intrinsic pathway			
P53	Triggers the intrinsic pathway	31	(31;71;73-76;76;90;113;113-136)*
Bcl-2	Anti-apoptotic Bcl-2 family member	38	(29;31;35;73;83-90;94;113;114;116;118;123;125;128;129;132;134;137-151)
Bcl-XL	Anti-apoptotic Bcl-2 family member	1	(90)
Bag1	Enhancing anti-apoptotic function of Bcl-2	2	(90;151)
Apaf-1	Formation of the apoptosome	8	(90;113;118;121;152-155)
Bax	Pro-apoptotic Bcl-2 family member	8	(90;123;123;146;147;156-159)
Bad	Pro-apoptotic Bcl-2 family member	1	(160)
Bid	Pro-apoptotic Bcl-2 family member	2	(90;160)
Bim	Pro-apoptotic Bcl-2 family member	1	(161)
Noxa	Pro-apoptotic Bcl-2 family member	1	(161)
Puma	Pro-apoptotic Bcl-2 family member	1	(161)
Caspase-8	Initiator Caspase of intrinsic pathway	2	(60;155)
Extrinsic pathway			
FasR	Death receptor	2	(56;57)
TrailR1	Death receptor	2	(57;59)
TrailR2	Death receptor	2	(57;59)
DcR1	Decoy death receptor	1	(57)
DcR2	Decoy death receptor	1	(57)
FasL	Death receptor ligand	1	(56)
TRAIL	Death receptor ligand	3	(57;59;60)
c-Flip	Inhibitor of extrinsic apoptosis induction	2	(56;60)
Caspase 9	Initiator Caspase of the extrinsic pathway	1	(155)
Cascade regulator			
IAP	Inhibitor of the Caspase cascade	3	(90;162;163)
Survivin	Inhibitor of the Caspase cascade	8	(35;90;94-97;99;108)
Tucan	Inhibitor of the Caspase cascade	1	(90)
XIAP	Inhibitor of the Caspase cascade	2	(90;164)
Smac/Diablo	Inhibitor of the IAP's, pro-apoptotic	3	(90;163;165)

Listed are biomarkers related to the pathway of apoptosis that emerged from the review of the literature. The biomarkers were studied by immunohistochemical analyses and their expression was related to clinical outcome in colorectal cancer patients. For each marker, the number of publications in which these markers were studied and their primary function within the pathway of apoptosis are listed.

* The systematic review of Munro et al., in which the available literature on the prognostic value of p53 expression until 2005 was reviewed extensively, was used as a starting point for our search in of the IHC literature on p53 expression. We therefore only included studies published since this review.

mediated apoptosis may, therefore, be a function of the ratio of DcR to DR. If there is significant upregulation of the DcRs or downregulation of the DRs, TRAIL will bind to the DcRs instead of the DRs, and the apoptotic signaling is interrupted (52). Secondly, cellular Flice-Like Inhibitory Protein (c-FLIP) is, similarly to FADD, a DD-containing protein and can competitively bind to FADD in the DISC formation process instead of the DD domain of the DRs (figure 1). This protein, particularly in the c-FLIP_L isoform, shows strong structural similarities to pro-caspase-8, and might be a potentially strong inhibitor of the extrinsic apoptotic pathway. There are two other features that are unique to the extrinsic pathway, but contribute considerably to the complexity of its regulation. The first feature is an indirect link with the intrinsic pathway, which can be activated through the formation of tBid, a truncated form of the BID protein. In a subset of cells known as type II cells, DISC formation occurs less frequent, resulting in less caspase-8 activation and subsequently truncation of the Bid protein into tBid (53). tBid induces oligomerization of Bax or Bad, upon which the mitochondria release cytochrome c, this eventually induces apoptosis further down the intrinsic pathway. Because of the mitochondrial involvement, the regulation of apoptosis in Type II cells is subject to regulation by the Bcl-2 family proteins. This regulation, which will be discussed in detail in this review, provides the cell with an apoptosis-evading mechanism such as downregulation of DR expression that may occur in a cell during tumorigenesis (54). A second feature unique to the extrinsic pathway is explained by the so-called 'Fas-Counterattack hypothesis' (55). In normal tissue homeostasis, the Fas/FasL-induced extrinsic pathway of apoptosis plays a major role in immune surveillance. Activated T lymphocytes express FasL and upon recognition of a tumor cell as a target (via MHC-presented peptides on the tumor cell surface), a tumor cell expressing the FasR may be eliminated by induction of apoptosis. However, it is known that tumor cells can also express FasL and thus are able to counterattack cells from the immune system (55). By downregulation of FasR expression as well as by upregulation of FasL expression, tumor cells can escape immune surveillance. In summary, the key players of the extrinsic pathway are the DRs, specifically TRAILR1, R2 and FasR and their ligands. The way tumor cells try to disrupt signaling through these DRs to overcome apoptosis has been widely studied in many types of cancers. Our search identified 12 studies in which one or more of these DRs and their ligands were studied. In 5 of the 12 studies, one of the DR pathway-related markers (FasR, FasL, TRAILR1 and TRAIL) was found to be of significant prognostic value (56-60) (table 3). Hypothetically, based on the biology of the process of tumorigenesis, downregulation of expression of the DRs or upregulation of expression of their ligands would indicate a more aggressive tumor type, and hence worse clinical outcome parameters. Interestingly, most studies reported that upregulation of the expression of Fas and TRAIL was significantly related to worse outcome parameters. The expression of FasL and FasR was studied by both Korkolopoulou *et al.* and Strater *et al.* (56;58). In the smaller study by Korkolopoulou *et al.* involving 90 patients, normal cells did not express FasL, but tumor cells showed significant upregulation, which was related to a significantly lower overall

Table 3

Extrinsic pathway of apoptosis

Overview of the literature on statistically significant, prognostic markers within the extrinsic part of the apoptotic pathway

Marker	Reference	Population size	Tumor Type	Disease Stage	Expression	Outcome parameter	Hazard Ratio	p-value
FasR	Strater(58)	128	Colon	II-III	Up	DFS	0.4	0.034
FasL	Korkolopoulou(56)	90	Colon	I-IV	Up	OS	3.491	0.005
TRAILR1	Van Geelen(59)	376	Colorectal	III	Up	REC	2.19	0.03
						OS	2.22	0.04
	Strater(57)	129	Colon	II-III	Up	DFS	2.59	0.003
TRAIL	McLornan(60)	253	Colorectal	II-III	Up	OS	1.210	0.026

Abbreviations: DFS (Disease Free Survival), OS (Overall Survival), REC (Recurrence), Up (Upregulation of the expression of the marker). This table provides the references of the studies that describe the prognostic value of FasR, FasL, TRAILR1, or TRAIL, identified in this review. For each marker the important study characteristics are listed.

survival (OS) (56). Tumor cells also showed expression of the Fas receptor with a mainly cytoplasmatic and granular staining pattern. According to the authors, this indicates that although Fas receptor was present, it had no true functional properties. Therefore, according to the authors, the seemingly contradictory result of a worse outcome despite upregulation of the DR expression could be explained by the Fas-Counterattack hypothesis. In the second study by Strater et al., overexpression of Fas receptor correlated with a significantly better disease free survival (DFS) (58). Unfortunately, this study did not describe the exact location of FasR expression in the cell. Therefore, it is difficult to determine whether their results confirm Korkolopoulou results or actually oppose them. We were therefore not able to determine whether the Fas-Counterattack hypothesis has true clinical value in CRC.

With respect to DR4 and its ligand TRAIL we could identify three studies reporting on the prognostic value of these biomarkers in CRC (57;59;60). In all studies, upregulation of expression of DR4 or its ligand were related to worse outcome parameters such as higher levels of recurrence and shorter OS. This apparent contradiction with expectations based on biology of tumorigenesis can, according to Van Geelen *et al.*, be explained by the fact that DR4 is also known to have effects on cell proliferation through the activation of nuclear factor kappa B (NF- κ B), as described in a number of studies (60-63).

In conclusion, we were able to identify five studies that report on FasL, FasR, TrailR1, or TRAIL 2 as significant prognostic markers in colorectal cancer patients. Conclusions varied, which might be due to differences in patient selection and/or study methods. However, more importantly, their conclusions were in contradiction with what is expected based on the biology of the apoptosis pathway. This can be explained by the fact that the functionality of this extrinsic part of the apoptotic pathway in the included studies was only investigated using changes in protein expression patterns of the tumor cells. The involvement of the immune system

was not considered in the majority of these studies. The process of tumorigenesis attracts many cells that are part of the immune system into the tumor micro-environment. The presence of these cells such as activated CD8+ T cells or Foxp3+ regulatory T cells has been shown to be of prognostic relevance in CRC (64;65). Moreover, activated T cells produce CD95 ligand and can hereby trigger apoptosis in target cells such as tumor cells (66). As described above, some tumors cells might be able to counteract this mechanism and remove attacking antitumor T cells by increasing their own CD95L expression. However, this counterattack theory has not yet been conclusively demonstrated in vivo. Therefore, until additional preclinical, exploratory research has been performed to clarify how the pathway of apoptosis and the immune system interact, none of the related markers appear suitable for clinical prognostic application.

P53 tumor suppressor gene and the intrinsic pathway of apoptosis

The p53 tumor suppressor gene, likely the most well known protein within the intrinsic pathways of apoptosis, encodes for a transcription factor that regulates the expression of genes involved in the pathway of apoptosis, as well as angiogenesis, cell cycle progression and genomic maintenance (67;67;68). Within the intrinsic pathway it exerts its function at the beginning of the intrinsic apoptotic pathway. It causes the cell cycle to arrest at the G1 phase in response to DNA damage; in case the DNA damage turns out to be irreparable, the p53 protein will activate the appropriate cellular signaling cascades to execute apoptosis. In 50% of human colorectal cancers p53 is absent or mutated, which has major implications for the execution of apoptosis in colorectal cancer (69). Mutations in p53 can be determined using IHC because mutated proteins accumulate in the nucleus due to their increased half-life (70). Different mutations have different effects and can implicate either loss or gain of function of the p53 protein. Two research groups carried out major systematic reviews on the relationship between p53 abnormalities and outcome in colorectal cancer patients (71;72). Munro *et al* reviewed a total of 168 IHC-based studies as well as mutation-based studies (71). Russo *et al*. pooled data from studies analyzing p53 DNA mutations only (72). Together, these studies reported on p53 expression and mutations in relation to survival in 18,766 patients. Their key finding was that abnormal expression of p53, detected using IHC, was related to an increased risk of death. They concluded that mutations in exon 5 were associated with an adverse outcome, predominantly in proximal, right-sided tumors. Both studies suggested an impact of mutated p53 on clinical outcome, though this relationship was only modest despite the overwhelming amount of data analyzed. The results of these studies were taken into account in the review of prognostic biomarkers in CRC by the ASCO Clinical Oncology's Tumor Marker Expert Panel in 2006. The ASCO panel's recommendation was that with current methods of detection, using either mutation analysis or IHC, p53 status was a poor guide for prediction of prognosis in colorectal cancer patients (19). Since the published literature on IHC-based p53 studies until 2005 has already been thoroughly reviewed by Munro *et al*., we limited our search to reports that

Table 4

P53 as a clinical prognostic marker

Overview of the literature on p53 expression in which p53 was proven to be an independent prognostic indicator of outcome in CRC patients

Reference	Population size	Tumor Type	Disease Stage	Outcome parameter	Hazard Ratio	p-value
Noske(76)	116	Colorectal	III	OS	-	0.048
Munro*(71)	12257	Colorectal	I-IV	OS	1.32	<0.0001
Torsello(73)	58	Colorectal (<40 years age)	I-IV	OS	2.48	0.046
Lim(75)	213	Colorectal	I-III	OS	1.843	0.028
Jurach(74)	83	Rectal	II-III	OS DFS	2.32 2.45	0.01 0.04

Abbreviations: OS (Overall Survival), DFS (Disease Free Survival).

This table provides the references of all the studies that were identified in this review to report on upregulation of p53 expression as a statistically significant, independent predictor of outcome in CRC patients.

*This article describes a review of multiple studies on the prognostic value of upregulated p53 expression determined with immunohistochemistry (IHC) in colorectal cancer patients.

have been published since. We identified an additional 30 studies reporting on the expression of p53 determined with IHC that were published after Munro's review in 2005. Four of these 30 papers confirmed the results of Munro *et al.* and claimed p53 to be a significant, prognostic marker in colorectal cancer (73-76). Table 4 provides an overview of their study characteristics. From this table, it can be concluded that although p53 has been widely studied, the studies reporting on statistically significant prognostic relevance show differences in population size, tumor type selection, and disease stage selection. When we examined these publications in more detail, we also noticed that they varied in their IHC methods. Previously, a comparative study by Baas *et al.* has demonstrated the monoclonal antibody DO7 to be superior over 5 other antibodies in the detection of the p53 gene protein in archival tissue of colorectal carcinomas (77), suggesting that this antibody should be used as a gold standard for IHC of p53. This particular antibody was used in 3 out of the 4 studies listed (73;75;76). Furthermore, the sample sizes of all studies were rather small at an average of 103 patients; and a closer look at these populations showed that most also seemed highly selected on clinical parameters. For example, Jurach *et al.* only included stage II and III rectal cancer patients and the small cohort of Torsello *et al.* consisted only of patients under age 40 (73;74). The only relatively large study by Lim *et al.* that analyzed the results of 231 stage I, II and III CRC patients, showed a correlation between upregulation of p53 expression and poor OS (75). This correlation was more pronounced in their stage III patient selection, but disappeared when only the adjuvant-treated patients were analyzed. Their results appeared to be confirmed by the study of Noske *et al.* (76). However, in this study the prognostic value of p53 was only present in a multivariate analysis when expression was analyzed in combination with p21 expression, a major

downstream cell cycle inhibitor. The expression of p53 alone in univariate analysis was only borderline significant at a p-value of 0.045 in this cohort of 116 stage II/III patients. As a single marker, p53 expression showed no independent statistical significance with respect to the prediction of outcome. Hence, neither of the four studies, although they claimed p53 to be an independent prognostic predictor of outcome in CRC, were able to add more significance to the conclusions drawn by Munro *et al.* (71). Therefore, their results are likely not sufficiently significant to alter the recommendations of the ASCO of 2006 with respect to the applicability of p53 as a prognostic biomarker in colorectal cancer (19).

The Bcl-2 family members and the intrinsic apoptotic pathway

Downstream of p53, the mitochondria play a major role in the initiation and execution of the intrinsic pathway of apoptosis. The B-cell CLL/Lymphoma 2 (BCL-2) family members are mainly responsible for regulating the intrinsic pathway and can be categorized into two groups. The first group consists of anti-apoptotic proteins that are structural and functional homologs of Bcl-2. The most important members of this group are Bcl-2 itself and its splice variant Bcl-2 XL (78;79). They are mainly bound to the mitochondrial outer membrane (MOM) by their transmembrane (TM) domain, where they stabilize the MOM to prevent cytochrome c release into the cytosol of the cell under normal homeostatic circumstances (80). Therefore, they can be considered anti-apoptotic proteins (81).

The second group of Bcl-2 family members has pro-apoptotic capacities. These members include Bcl-2 associated X protein (BAX) and proteins such as Bad, Bid, Bim, Bik, Noxa and Puma, which are, based on their structure, also known as BH3-only proteins (78). These proteins are usually bound to the cytoskeleton or cytosol, but upon stimulation they interact with and inhibit their anti-apoptotic counterparts such as Bcl-2 (78). The relative ratio or balance between the expression of both groups of Bcl-2 family members will determine whether stimulation of the intrinsic pathway of apoptosis results in apoptosis as is graphically pointed out in Figure 1. If the pro-apoptotic factors predominate, cytochrome c will be released into the cytosol where it binds to the apoptosis-activating factor 1 (Apaf-1) to form an apoptosome. More downstream in the apoptotic pathway, this apoptosome will form a complex together with an initiator caspase, caspase-9. This caspase will subsequently activate the executioner caspases, caspase-3 and -7 (78;82). Deregulation of apoptosis during tumor development can be caused by a disturbance in the homeostatic balance of the Bcl-2 family members.

Our search resulted in 55 studies describing the prognostic relevance of markers related to the Bcl-2 protein family. In the majority of these studies, 38 in total, the expression of Bcl-2 was studied using IHC. In only 9 out of these 38 studies a statistically proven prognostic relevance of this marker could be established (table 5) (73;83-90). The 9 studies generally used the same methods to determine Bcl-2 expression and all but one were performed on whole paraffin-embedded tissue sections. Furthermore, they were very consistent in their conclusions: in all studies upregulation of Bcl-2 was related with better survival, as shown for either

Table 5

Bcl-2 as a clinical prognostic marker

Overview of the studies reporting on Bcl-2 as significant, prognostic marker within the pathway of apoptosis.							
Reference	Popula- tion size	Tumor Type	Disease Stage	Expression	Outcome parameter	Hazard Ratio	p-value
Buglioni (83)	171	Colorectal	I-IV	Down	DFS	5.61	0.0009
					OS	5.21	0.0063
Schwander (84)	160	Colorectal	I-III	Up	REC	-	0.0242
Chatla(85)	158	Colorectal	II-III	Down	OS	-	0.0012
Sinicrope(89)	63	Colorectal	I-II	Up	RFS	0.23	0.04
					OS	0.17	0.03
Krajewska(90)	154	Colon	II	Up	RFS	0.45	0.04
					OS	0.25	0.0009
Leahy(86)	102	Colorectal	I-III	Up	OS	0.5	0.005
Ilyas(87)	66	Colorectal	II	Up	REC	0.77	0.02
Torsello(73)	58	Colorectal (<40 years age)	IV	Down	OS	3.02	0.015
Elkablawy(88)	52	Colorectal	I-IV	Up	OS	-	0.016

Abbreviations: DFS (Disease Free survival), OS (Overall Survival), REC (Recurrence), RFS (Recurrence Free Survival), Up (Upregulation of marker expression), Down (Downregulation of marker expression).

Table 5: A list of 9 studies, identified by this review, describing a statistically significant, prognostic effect of Bcl-2 in colorectal cancer patients. For each maker, the important study characteristics are listed.

disease free, overall, or recurrence free survival. This is contradictory to what is assumed given the anti-apoptotic function of Bcl-2: upregulated Bcl-2 expression would be more likely to be a marker of a more aggressive tumor phenotype. The most plausible explanation for this paradoxical finding is the fact that Bcl-2 not only has an anti-apoptotic function, it can also exert a distinct negative influence on cell cycle progression which can eventually slow down tumor growth. This may explain the survival benefit for several patients with upregulated Bcl-2 expression (83-85;89). Whether the anti-cell cycle progression or the anti-apoptotic role of Bcl-2 predominates during tumorigenesis may depend on disease stage. In early carcinogenesis, the anti-apoptotic function of Bcl-2 plays a large role, causing genetic alterations to accumulate. In later stages, Bcl-2 functions more as a cell cycle progression inhibitor, lowering the rate of tumor proliferation. This hypothesis is supported by the inverse correlation between Bcl-2 and the percentage of cells in S-phase found by Buglioni *et al.* (83). The studies describing the prognostic relevance of Bcl-2 would therefore be more informative if disease stage would and the expression of the other family members were taken into account. This would provide us additional insight in the biologic function and effects on the apoptotic pathway of Bcl-2, which will tremendously improve the interpretation of the results.

Inhibitors of apoptosis family proteins and the execution of apoptosis

The actual apoptotic cell death machinery, responsible for the execution of apoptosis and resulting in the morphologic features characteristic for apoptosis, consists of a very complex cascade of interacting proteins. The key components include the caspase proteins, as described above. At many levels regulation takes place to ensure appropriate functioning of the caspase machinery. The key regulators of the caspase cascade are the inhibitors of apoptosis proteins (IAPs) that exert their function through binding of activated caspases. So far, eight IAPs have been identified in mammals, the most well-known being livin, X-linked inhibitor of apoptosis (XIAP) and survivin (26;91;92). All IAP family proteins have one or several specific Baculoviral IAP repeats (BIRs). They require at least one BIR to exert their anti-apoptotic function. The function of the IAPs is also strictly regulated, by their own set of inhibitors such as Smac/Diablo and Omi/HtrA2 (26). Under normal circumstances, when apoptotic stimuli are present, cells release Smac/DIABLO from their mitochondria into the cytosol where the complex exerts its pro-apoptotic effect by interacting with the IAPs in order to release bound caspases into the cytosol (93). The most frequently studied IAP in our search results was survivin, likely because the role of survivin in apoptosis has been the subject of controversy over the last few of years. Of the 8 studies describing the prognostic effects of survivin, 4 demonstrated a statistically prognostic effect of survivin (35;90;94-99) (table 6). Initially it was thought that survivin and the other IAPs selectively bind active caspase-3/ -7 and -9, promoting their degradation and thereby inhibiting apoptosis (100). Survivin, however, lacks the structural motif to bind to caspases and likely only inhibits activated caspase-9 with the help of XIAP (101-103). In contrast to other IAPs, survivin is undetectable in normal adult tissues, but abundantly expressed in transformed cell types and a variety of human cancers, such as cancers originating in the colon, stomach, pancreas, lung, prostate, and breast (104). Although all four studies were able to show a significant relation between survivin expression and clinical outcome, the direction of this effect was not the same. When looking at these studies in detail it was noticed that they did not apply the same methods of analysis of the IHC results. This is of importance because survivin can be expressed in two cellular compartments: either in the cytoplasm or in the nucleus with different functions (98;105;106). In general, survivin is known to be involved in the regulation of cell viability as well as in the regulation of cell division. It is hypothesized that the nuclear subset is involved in controlling cell proliferation and the cytoplasmic pool is more involved in regulating cell survival (107). Sarela *et al.* found a relationship between survivin expression and a shorter DFS when scoring mainly the cytoplasm for survivin positivity (94). Ponnelle *et al.* showed a positive influence of both cytoplasmic and nuclear expression on survival in a very small patient population of only 46 patients. This only reached statistical significance for the cytoplasmic group (95). Fang *et al.* showed a negative effect of survivin expression on OS, disease recurrence, and the development of liver metastasis (108). The same was true for the study by Sprenger *et al.* in which pretreatment biopsies of rectal cancer patients were analyzed for their survivin

Table 6
Survivin as a clinical prognostic marker

Overview of the studies reporting on Survivin as significant, prognostic marker within the pathway of apoptosis							
Reference	Popula- tion size	Tumor Type	Disease Stage	Expression	Outcome parameter	Hazard Ratio	p-value
Sarela(94)	49	Colorectal	II	Up	OS	9.1	0.03
Ponnelle(95)	46	Colorectal	I-IV	Up	OS	0.35	0.045
Fang(108)	630	Colon	I-IV	Up	OS	1.63	0.018
Sprengrer(99)	116	Rectal	II-III	Down	DFS	-	0.038

Abbreviations: OS (Overall Survival), Up (Upregulation of marker expression), Down (Downregulation of marker expression).
This table provides the characteristics of all of the studies, identified by this review, that describe a significant prognostic effect of survivin expression determined by immunohistochemistry in colorectal cancer patients. For each marker, the important study characteristics are listed.

expression (99). In this study, low pre-treatment expression was related to a significantly better DFS. Unfortunately, neither of the groups elaborated on the specific location in the cell at which they scored survivin expression. The image of a tissue microarray (TMA) core that was immunohistochemically stained for survivin expression provided in the publication by Fang *et al.* suggests that the staining pattern was predominantly cytoplasmatic (108). In conclusion, it seems that localization of survivin expression is in fact of great importance as it is probably related to the protein function and hence should be taken into account in future studies. This may be applicable to all of the other IAP family members as none have been studied widely with standardized scoring methods in large series.

DISCUSSION

This review gives an overview of the literature published on IHC-based prognostic biomarkers related to the pathway of apoptosis in colorectal cancer between January 1998 and June 2011. Particularly, we discussed those markers that were proven to be of independent, statistically significant, prognostic value by placing them in the context of their function within the apoptotic pathway. Based on this biological background information, we then analyzed the conclusions drawn by the authors of the studies included to see whether their conclusions could provide valuable grounds to proceed investigating these markers for prognostic clinical application. The markers we discussed are all major regulatory players in this pathway such as p53, the Fas receptor and the DR4 with their respective ligands, the Bcl-2 protein family, and survivin. In general, we concluded that none qualified as a single prognostic biomarker for colorectal cancer patients, despite the fact that it has been well-established that the outcome of the pathway of apoptosis is of prognostic value (29-37;99). Based on the information derived from all of the

studies discussed, we postulate several explanations for this lack of sufficient clinical prognostic significance for individual markers of the apoptotic pathway. First, study characteristics of investigations of one specific biomarker varied widely. This sometimes provided a marker with prognostic significance, but only in a highly selected group of patients. For example, in the case of p53, some of the studies were able to reproduce the results of the review of Munro *et al.* (71), but only when a selected patient cohort was studied which consisted of patients with better survival rates after surgery, as was the case in the study by Lim *et al.* (75). In this study, the prognostic value of p53 was only present in the stage II/III cohort when both adjuvant and non-adjuvant treated patients were included, which were probably the patients with such good outcome perspectives after surgery that adjuvant treatment was deemed unnecessary. The prognostic value disappeared when only the adjuvant-treated cases were analyzed. This makes the applicability of p53 as a general prognostic marker at time of diagnosis and treatment allocation questionable for the entire colorectal cancer patient population. Second, well-standardized IHC protocols were applied for none of these markers. Possibly even more importantly, there seemed to be no standardized methods for quantifying expression level or a specific location in the cell at which the expression of a marker should be evaluated. This may greatly influence the interpretation of the results of these studies, as for instance the specific location of expression in a cell might be directly related to its biological function and thereby to its effect on the outcome of the apoptotic pathway and patient prognosis. Caution should be taken in the method of quantification, particularly for FasR and survivin expression. In the case of the FasR expression, a high level of expression found in the cytoplasm but not on the cells membrane can indicate the presence of decoy receptors (56). This might be an indication that the so-called Fas-Counterattack hypothesis is indeed true, making it essential to evaluate the subcellular location of expression (55). In addition, in the case of survivin, staining location seemed to be of great importance as both nuclear and cytoplasmic expression showed a different relation with outcome in the studies reviewed (94;95;99;108). We concluded that location of expression might implicate different biological functions of the same protein. Third, we showed that the pathway of apoptosis is strictly regulated at several levels by both stimulatory and inhibitory proteins that highly interact with each other. Using only one marker to describe the outcome of these interactions, therefore, seems inappropriate. To complicate matters even more, the function of a protein might differ depending on progression of the process of tumorigenesis as was shown for Bcl-2 expression (83). Intervention of key proteins of apoptosis in other pathways makes cellular outcome unpredictable when using expression of single proteins as prognostic biomarkers.

Overall, we can conclude that studying outcome of the pathway of apoptosis, or deciding on a patient's prognosis and treatment, using single markers seems inappropriate given the complexity of this pathway. We already highlighted the delicate balance that exists between expression and effects on apoptosis of the members of the Bcl-2 pathway and of the IAP's and their inhibitors (78;83). To interpret the

effect of expression of single proteins without the knowledge of the expression status of any of the others involved seems inappropriate. Therefore, we would like to propose suggestions to improve the clinical applicability of these markers. Most importantly, we suggest that the apoptotic profile of a tumor should be determined rather than expression of single markers. This should include several markers that together represent the outcome of all regulatory thresholds within the pathway. An apoptotic profile would better represent the true function of the markers involved and provide insight on the outcome of the apoptotic pathway in individual tumors; thus, an apoptotic profile fulfills the need for prognostic biomarkers in colorectal cancer (22).

In the literature studied for this review, several authors already made an attempt to establish such a multi-marker apoptotic phenotype (59;60;83;99). However, in these studies, the selection of biomarkers was based on the markers that showed some prognostic relevance in their series as a single biomarker without any respect to the biology of the pathway. In addition, in most cases the markers used in these 'multi-marker phenotypes' all belonged to just one sub-regulatory unit of the pathway. For example, Van Geelen *et al.* and McLornan *et al.* both studied multiple markers with respect to the DRs, but did not include any of the more downstream regulator proteins (59;60). Furthermore, in many of the studies the statistical methods used to analyze the results of these multi-marker phenotypes lack power. A solution to this problem might be to approach the data as one would do in the case of gene expression array data by performing hierarchical clustering in order to develop a profile (109). Until then, the biomarkers that will eventually make up this apoptotic profile remain to be determined. In the meantime, we suggest that further studies focus on analyzing the clinical relevance of not only the outcome of (de) -regulation of the apoptotic pathway in colorectal cancer but also on the outcome of the (de) -regulation of the pathway of proliferation. Under normal circumstances a key factor in tissue homeostasis is the balance that exists between the level of cell death and the level of cell proliferation (110;111). Deregulation of either of these pathways can therefore cause disturbance of this balance, which may result in and maintain tumorigenesis. This hypothesis has been studied previously with success in a cohort of 100 colorectal cancer patients in which an AI:PI ratio was determined (112). This Apoptotic Index: Proliferation Index, based on M30 IHC for the level of apoptosis and Ki67 IHC for the proliferation index, was significantly to patient outcome. It remains to be seen if studies in larger patient populations will confirm these results.

In conclusion, to determine the prognostic relevance of biomarkers of the pathway of apoptosis in colorectal cancer using immunohistochemistry, multiple markers that together reflect the apoptotic status in individual tumors should be studied together. The introduction of such a multi-marker apoptotic phenotype or -profile into clinical practice demands standardization of technical assays and quantification methods. For future studies, therefore, we recommend to consider the full pathway when starting the exploratory phase towards the discovery of new biomarkers in colorectal cancer related to outcome of the apoptotic process.

Moreover, we recommend to apply hierarchical clustering-based statistical analysis and use knowledge of the biology of the pathway to identify promising markers. And furthermore, we recommend to even consider to take in to account markers representing the pathway of proliferation when studying the prognostic effects of the apoptotic pathway in colorectal cancer. These measures will lead to multi-marker profiles that can then be validated in large retrospective studies and can ultimately be introduced into clinical practice.

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SUPPLEMENTARY DATA

List of abbreviations (in order of appearance)

IHC	Immunohistochemistry
CRC	Colorectal Cancer
ASCO	American Association of Clinical Oncology
EGTM	European Group on Tumor Markers
Bcl-2	B-cell Lymphoma 2
P53	Protein 53
TRAILR1	Tumor necrosis factor Related Apoptosis Inducing Ligand Receptor 1
UICC	International Union Against Cancer
TNM	Tissue Node Metastasis
ASCO-TEMP	American Association of Clinical Oncology Tumor Markers Expert Panel
DNA	Deoxyribonucleic Acid
UV	Ultraviolet
IAP	Inhibitor of Apoptosis Protein
DR	Death Receptor
FasR	Fas Receptor
TRAILR2/R3/R4	TRAIL Receptor 2/3/4 (see TRAILR1)
FasL	Fas Ligand
c-Flip	Cellular FADD Like Interleukin 1 beta-converting Enzyme Inhibitory Protein (see FADD)
TNFR	Tumor Necrosis Factor Receptor
DD	Death Domain
FADD	Fas-Associated Death Domain
DISC	Death Inducing Signalling Complexes
DcR1/2	Decoy Receptor 1/2
MHC	Major Histocompatibility Complex
OS	Overall Survival
DFS	Disease Free Survival
NF- κ B	Nuclear Factor kappa-light-chain-enhancer of activated B cells
CD8	Cluster of Differentiation 8
CD95	Cluster of Differentiation 95
T cell	Thymus cell
CD95L	CD 95 ligand (see CD 95)
Bcl-2 XL	Bcl-2 Extra Large (see Bcl-2)
MOM	Mitochondrial Outer Membrane
TM	Transmembrane
BAX	Bcl-2 Associated X protein (see Bcl-2)
BAD	Bcl-2 Associated Death Promoter (see Bcl-2)
Bim	Bcl-2 like interacting mediator of cell death (see Bcl-22)
Bid	BH3 interacting domain death agonist (see BH3)

Puma	P53 upregulated modulator of apoptosis
TUCAN	Tumor-upregulated CARD containing antagonist of caspase 9 (see CARD)
CARD	Caspase Recruitment Domain
BH3	Bcl-2 Homology 3 (see Bcl-2)
Apaf-1	Apoptosis-Activating factor 1
S-phase	Synthesis phase
XIAP	X-linked IAP (see IAP)
BIR	Baculovirus IAP Repeat (see IAP)
SMAC	Second Mitochondria-Derived Activator of Caspases
DIABLO	Direct IAP Binding protein with Low pl. (see IAP)
HtrA2	High temperature requirement protein A2
TMA	Tissue Micro Array
N	number of patients in study population
Bag1	Bcl-2 associated Athanogene 1
REC	Recurrence
RFS	Recurrence Free survival

CHAPTER 3

Combined analysis of biomarkers of proliferation and apoptosis in colon cancer; an immunohistochemistry based study using tissue micro array

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Accepted in International Journal of Colorectal disease. 2014

ABSTRACT

Background: Disturbance of the balance between proliferation and apoptosis is an important hallmark of tumor development. The goal of this study was to develop a descriptive parameter that represents this imbalance and to relate this parameter to clinical outcome in all four stages of colon cancer.

Material and methods: The study population consisted of 285 stage I-IV colon cancer patients of which a tumor tissue micro array (TMA) was available. Sections of this TMA were immunohistochemically stained and quantified for presence of Ki67 and cleaved caspase-3 tumor expression. These results were used to develop the combined apoptosis proliferation (CAP) parameter and correlated to patient outcome.

Results: The CAP parameter was significantly related to clinical outcome; patients with CAP ++ (high level of both apoptosis and proliferation) showed the best outcome perspectives (Overall Survival (OS), $p=0.004$ and Disease Free Survival (DFS), $p=0.009$). The effect of the CAP parameter was related to tumor microsatellite status, and indirectly to tumor location, where left-sided tumors with CAP + - (high level of proliferation, low level of apoptosis) showed a worse prognosis (DFS p -value 0.02) and right-sided tumors with CAP + - had a better prognosis (DFS p -value 0.032). With stratified analyses, the CAP parameter remained significant in stage II tumors only.

Conclusions: The CAP parameter, representing outcome of the balance between the level of apoptosis and proliferation, can be used as a prognostic marker in colon cancer patients for both DFS and OS, particularly in left sided, microsatellite stable tumors when TNM stage is taken into account.

INTRODUCTION

A key factor in tissue homeostasis, especially of the intestinal mucosa, is the balance that exists between the level of cell death and the level of cell proliferation (1-3). Two important hallmarks of the process of tumorigenesis are responsible for disturbance of this balance and therefore contribute to the initiation and maintenance of tumor growth and development (4;5). These hallmarks are: deregulation of the proliferative signaling pathway and deregulation of the pathway of apoptosis (5). Both result in either non- or malfunction of important enzymes or unrestricted release of growth-promoting signals that under normal circumstances are necessary to maintain tissue homeostasis (6-13). The level of cell proliferation and apoptosis can be studied with immunohistochemistry (IHC) taking advantage of all of the benefits of this technique, such as speed, routine availability, low costs, and high level of automation. The level of apoptosis can be evaluated through staining specifically the activated, cleaved form of the pro-apoptotic enzyme caspase-3 in the tumor cell cytoplasm. Caspase-3 is the final enzyme to become activated in the caspase cascade, which is the common pathway in the execution of apoptosis after the intrinsic and extrinsic apoptosis induction pathways converge. Therefore, the expression level of activated or cleaved caspase-3 should give a reliable measure of the level of apoptosis (14). The proliferation activity of a tumor can be estimated by determining the expression levels of specific cell cycle-related proteins also by using IHC. A widely used marker is the Ki67 antigen, which is expressed in nuclei during all cell cycle phases except during the G₀ phase (15). Previous studies showed contradicting results with respect to the relation of the level of apoptosis or proliferation in tumor resection specimens and patient outcome in colon cancer (16-23). We hypothesize that, because tissue homeostasis depends on the balance between cell death and proliferation levels, the level of disbalance between these processes indicate tumor aggressiveness. Therefore, combined and not separate analysis of these parameters might be of prognostic relevance in colon cancer patients.

In this study we determined both the level of tumor cell apoptosis and proliferation in resection specimens of a large cohort of colon cancer patients. We combined the results into one parameter and related this parameter to patient outcome data.

MATERIALS AND METHODS

Patients and tumors

The patient cohort consisted of 470 colorectal cancer patients treated with surgery for their primary tumor in the LUMC between 1991 and 2001. Clinico-pathological and follow-up data were collected retrospectively from hospital records and the hospital's oncology database. This research was performed according to the code of conduct for responsible use. Patients with a history of cancer other than basal cell carcinoma or cervical carcinoma *in situ*, patients that received radio- and/or chemotherapy treatment prior to resection, patients with multiple synchronous colon tumors, and patients with rectal cancers were excluded from the analysis (n=185). The entire study cohort consisted of 285 patients. Right-sided tumors were defined as those originating proximal to the splenic flexure and left-sided as those originating distal to the splenic flexure.

Primary Antibodies

The following antibodies were used in the immunohistochemical stainings: Mouse monoclonal antibody anti-Ki67 (DAKO Glostrup Denmark Art.M7240 clone MIB-1) to determine the level of tumor cell proliferation and rabbit polyclonal antibody anti-ASP-175 (Cell signaling Danvers, USA, Art.9661) was used for cleaved caspase-3 identification to determine the level of apoptosis.

Immunohistochemistry

Qualified pathologist evaluated the tumor material from all patients included for histo-pathological characteristics according to current standards during the routine hospital diagnostic process. Formalin-fixed paraffin-embedded tumor blocks of the primary tumor were collected from the pathology department. Sections were cut for haematoxylin and eosin staining, and representative tumor regions based on histological assessment were used for preparation of tumor tissue microarray (TMA) blocks. From each donor block, three 0.6 mm tissue cores were punched from tumor areas and transferred into a recipient paraffin block using a custom-made precision instrument.

Immunohistochemistry (IHC) staining was performed on 4 μ m sections that were cut from each receiver block and mounted on glass. For each primary antibody, all slides were stained simultaneously to avoid inter-assay variation. Tissue sections were deparaffinized and rehydrated. For antigen retrieval, 0.01 M EDTA buffer (pH 8) was used for 10 minutes at maximum power in a microwave oven for anti-Ki67. Citrate buffer 0.1M (pH 6) was used for anti-ASP-175. Endogenous peroxidase was blocked for 20 minutes in 0.3% hydrogen peroxide in methanol. Sections were incubated overnight with either anti-Ki67 or anti-ASP-175 at predetermined optimal dilutions. After 30 minutes of incubation with Envision anti-mouse (K4001; DAKO Cytomation, Glostrup, Denmark) or Envision anti-rabbit (K4003; DAKO Cytomation, Glostrup, Denmark), sections were visualized using diaminobenzidine solution. Tissue sections were counterstained with haematoxylin, dehydrated and

finally mounted in malinol. Sections with phosphate buffer saline (PBS) instead of primary antibody, which underwent the complete staining protocol served as negative controls.

Evaluation of immunohistochemistry

Microscopic analyses of Ki67 and cleaved caspase-3 expression was performed by two independent observers (M.S.R: 100% and T.C.A.: 30%) in a blinded manner. For Ki67, the percentage of tumor cells that showed nuclear staining was assessed. For determination of tumor cell apoptosis, the absolute number of caspase-3 expressing tumor cells in each tumor punch that showed cytoplasmatic and perinuclear staining was counted. The Cohen's Kappa for inter observer variability was 0.73 and 0.6 for Ki67 and cleaved caspase-3 respectively. Therefore, there was substantial agreement between the two observers and all scores were averaged. For analysis a cut-off at the median was chosen, dividing the samples in low (<27% positive tumor cells) or high nuclear Ki67 expression ($\geq 27\%$). The use of this percentage of positive cells as a cut-off point is supported by Fluge *et al.* (19). Cleaved caspase-3 was quantified into two categories of IHC cytoplasmatic tumor staining levels. Negative staining; implied no positive tumor cells in either of the three cores, in all other cases the staining was denoted as positive. Representative images of the Ki67 and caspase-3 staining are shown in Figure 1.

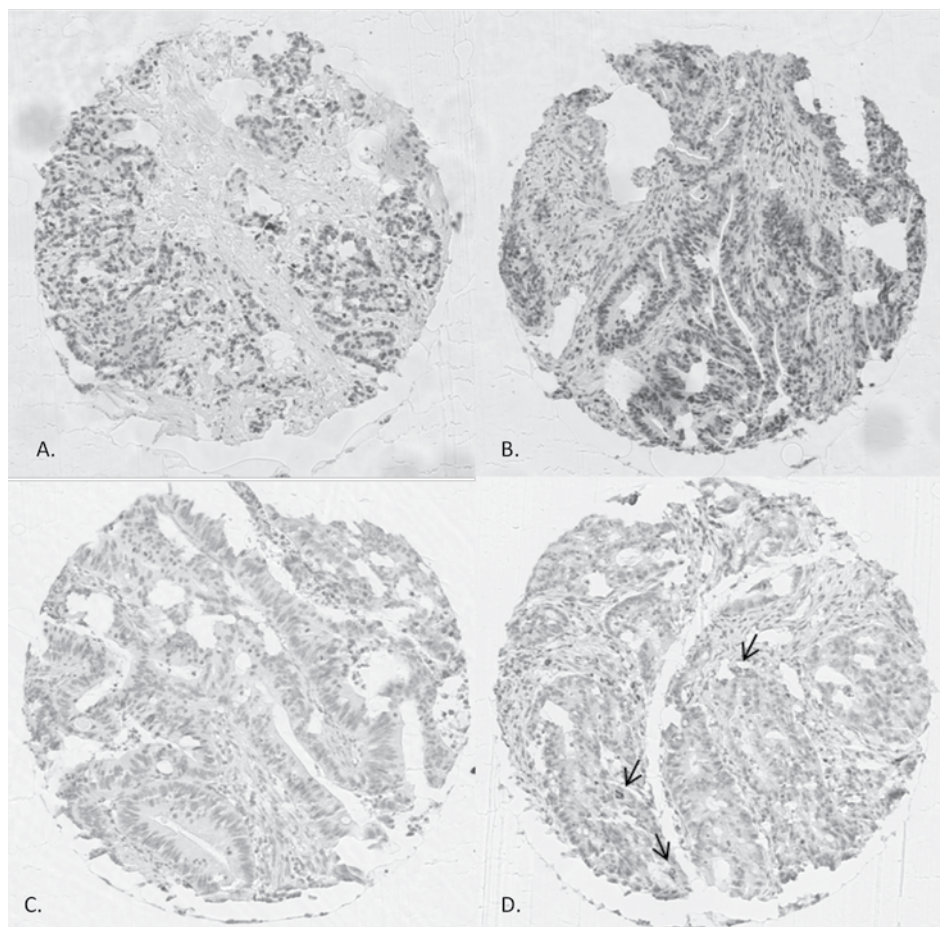
Determination of microsatellite stability status

DNA was extracted from 2mm tumor-cores. Paraffin was dissolved in xylene, tissue was rehydrated in ethanol (100%/70%) and dried for 10 minutes at 37°C. Nucleospin 96 Tissue kit (Machery-Nagel, Düren, Germany) was used for DNA extraction according to the manufacturer's protocol.

Microsatellite stability status was tested using the MSI Analysis System Version 1.2 (Promega, Mannheim, Germany) and interpreted by an experienced pathologist, as described previously (24).

Statistical Analysis

Statistical analyses were performed using the statistical package SPSS (version 17.0 for Windows; SPSS, inc). The Student's T-test, the Mann-Whitney U test and the Chi-squared test were used to evaluate associations between Ki67 or cleaved caspase-3 and various clinico-pathological parameters. The Overall Survival (OS) was defined as time between primary tumor resection and time of death and Disease Free Survival (DFS) as time between primary tumor resection and time of death or relapse of disease, whichever came first. The Kaplan-Meier method was used for calculation of survival probabilities and the Log-rank test for comparison of survival curves. Cox regression was used for univariate and multivariable analysis for OS and DFS. Significant variables (in univariate analysis) were included in multivariable analysis. For all tests, a p-value <0.05 was considered to be statistical significant.

**Figure 1**

Representative images of Ki67 and cleaved caspase-3 immunohistochemical staining in colon cancer tissues

(A) Ki67, tumor with low expression (B) Ki67, tumor with high expression (C) Tumor showing absence of cleaved Caspase-3 tumorcell expression and (D) Tumor showing presence of cleaved caspase-3 tumorcell expression, as indicated by the arrows.

RESULTS

Patient characteristics, and cleaved caspase-3 and Ki67 expression levels

The study cohort consisted of 285 patients. In 41 cases for Ki67, and 38 for cleaved caspase-3 the results of the IHC could not be analyzed due to loss of the tumor material during IHC or due to staining artifacts. Representative images of the biomarkers and their staining categories are shown in figure 1. The mean percentage of tumor cells expressing Ki67 in the tumor tissue cores was 29.2% with a median of 27.5%. For analysis we used the median as cutoff based on skewness of the data distribution. This resulted in 121 patients (49.6%) with tumors showing low expression level (below median) of Ki67 and ('low' tumor cell proliferation level) and 123 patients (50.4%) with high expression level (above median) of Ki67 ('high' tumor cell proliferation level). In 85 (34.4%) patients the tumor tissue cores showed no staining of cleaved caspase-3 and therefore no apoptotic activity of tumor cells. The remaining 65.6% of the samples showed positive staining and thus ongoing tumor cell apoptosis.

The clinico-pathological characteristics of the patient cohort and their relation to expression levels of the biomarkers are listed in table I. Interestingly, tumor location was significantly related to both cleaved caspase-3 expression level and Ki67 expression level. Microsatellite instability also showed statistical significance, but was only significantly related to cleaved caspase-3 expression and not to Ki67 expression. In the tumor samples without cleaved caspase-3 expression, 2.4% of the cases showed microsatellite instability vs. 16.7% in the tumors with expression of cleaved caspase-3 (p-value 0.004). Additional analysis showed in our patient cohort a strong, significant correlation between tumor location and microsatellite stability status with significantly more microsatellite instable tumors (MSI) located on the right side of the colon and the majority of the microsatellite stable (MSS) tumors located on the left side of the colon (70%), whereas this was only 8% in microsatellite instable tumors (MSI) ($p < 0.001$).

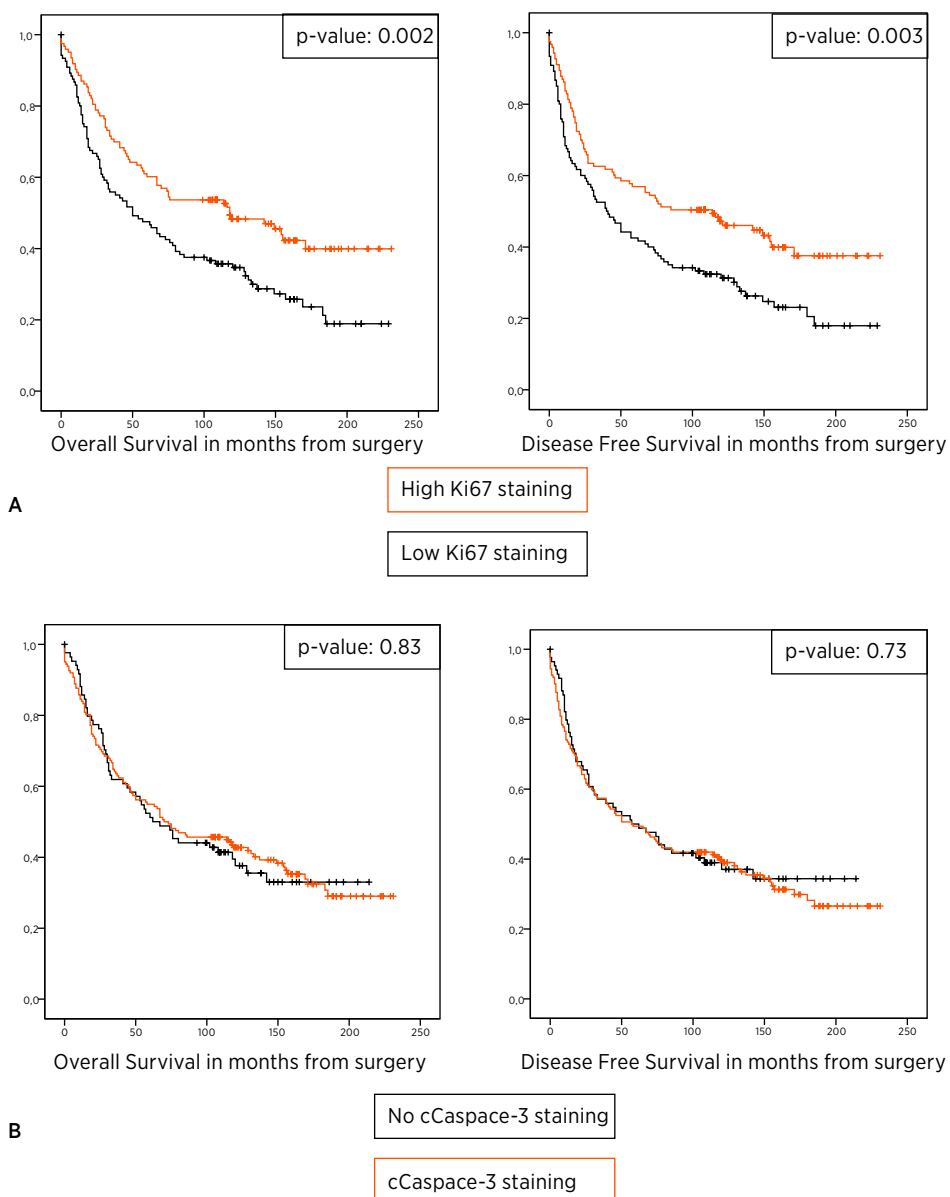
Relation of single marker expression with patient outcome

The level of tumor cell proliferation based on Ki67 expression level was significantly related to OS and DFS: high tumor expression level correlated significantly to a better patient OS and DFS (OS, Logrank p-value 0.002; DFS, Logrank p-value 0.003) (Figure 2). Tumor cell apoptotic level, as represented by cleaved caspase-3 expression, was not related to either OS or DFS (OS, Logrank p-value 0.83; DFS, Logrank p-value 0.73).

Table I
 Patient Characteristics of the Total Colon Cancer Cohort and stratified for HLA class I, HLA-EG and Foxp3+ expression

	Total Population (N=285)	Ki67 Absence N=121	Ki67 Presence N=123	cCaspase-3 Absence N=85	cCaspase-3 Presence N=162
Gender					
Male	137 (48.1%)	55 (45.5%)	65 (52.8%)	42 (49.4%)	80 (49.4%)
Female	148 (51.9%)	66 (54.5%)	58 (47.2%)	43(50.6%)	82 (50.6%)
Age (average)	65.7 (±13.3 SD)	67.3 (±11.6 SD)	64.2 (±13.6 SD)	66.5 (±12.6 SD)	65.6 (±12.9 SD)
TNM stage					
I	44 (15.4%)	14 (11.6%)	20 (16.3%)	16(18.8%)	18 (11.1%)
II	114 (40.0%)	43 (35.5%)	53 (43.1%)	31 (36.5%)	68 (42.0%)
III	74 (26.0%)	36 (29.8%)	30 (24.4%)	19 (22.4%)	47 (29.0%)
IV	48 (16.8%)	27 (22.3%)	19 (15.4%)	17 (20.0%)	29 (17.9%)
Unknown	5 (1.8%)	1 (0.8%)	1 (0.8%)	2 (2.4%)	0 (0.0%)
Grade			<i>p=0.004</i>		<i>p=0.017</i>
Moderate	145 (50.9%)	61 (50.4%)	63 (51.2%)	35(41.2%)	89 (54.9%)
Poor	23 (8.1%)	5 (4.1%)	16 (13.0%)	4 (4.7%)	18(11.1%)
Good	58 (20.4%)	32 (26.4%)	16 (13.0%)	22(25.9%)	26 (16.0%)
Unknown	59 (20.7%)	23 (19.0%)	28 (22.8%)	24 (28.2%)	29(17.9%)
MS Status					<i>p=0.004</i>
MSS	168 (58.9%)	77 (63.6%)	87 (70.7%)	61 (71.8%)	103 (63.6%)
MSI	30 (10.5%)	12 (9.9%)	14 (11.4%)	2 (2.4%)	27 (16.7%)
Unknown	87 (30.5%)	32 (26.4%)	22 (17.9%)	22 (25.9)	32 (19.8%)
Location			<i>p=0.023</i>		<i>p=0.011</i>
Right	110 (38.6%)	37 (30.6%)	53 (43.1%)	27 (31.8%)	64(39.5%)
Left	153 (53.7%)	78 (64.5%)	60 (48.8%)	57 (67.1%)	83(51.2%)
Unknown	22 (7.7%)	6 (5.0%)	10 (8.1%)	1 (1.2%)	15 (9.3%)

Abbreviations: MS Status; Microsatellite Status, cCaspase3; cleaved caspase 3.
 This table describes the baseline characteristics of the entire cohort of 285 patients in the first column. The Ki67 immunohistochemistry results could be analyzed in 244 cases and Ki67 expression (above the median of 27.5% expression level) was found to be present in 123 and absent in 121 patients. The other columns describe the relation of either Ki67 absence or presence in the tumor resection specimens to clinico-pathological parameters. The cleaved caspase 3 results were available for analysis in 247 patients. In this population 85 tumor samples showed no presence of cleaved caspase 3, expression was present in 162 tumor samples of patients. The fourth and fifth column describe the relation of either cleaved caspase 3 absence or presence to clinico-pathological parameters. Only significant ($p<0.05$) differences between marker expression as proven by χ^2 tests are displayed.

**Figure 2**

Abbreviations: cCaspase3; cleaved caspase.

Survival curves stratified for Ki67 and cleaved caspase-3 tumor expression in colon cancer (A) Kaplan Meier curves for OS and DFS in the study population of 285 colon cancer patients stratified for Ki67 tumor expression (B) Kaplan Meier curve OS and DFS in the study population of 285 colon cancer patients stratified for cleaved caspase-3 expression in their tumor sections.

Table II

Description of the CAP (Combined Apoptosis and Proliferation) parameter

CAP ++	Ki67 expression above the median and presence of cleaved caspase-3 IHC
CAP +-	Ki67 expression above the median and no presence of cleaved caspase-3 IHC
CAP -+	Ki67 expression below the median and presence of cleaved caspase-3 IHC
CAP --	Ki67 expression below the median and no presence of cleaved caspase-3 IHC

Abbreviations: IHC; immunohistochemistry.

This table provides the definitions of the CAP parameter. This parameter resulted from data combination on the tumor cell apoptotic level based on the cleaved caspase-3 immunohistochemistry results with the data on the tumor cell proliferation level based on the Ki67 expression levels as determined with IHC.

Combined analysis of tumor cell apoptosis and proliferation in relation to patient outcome

To analyze the effect of the balance between apoptosis and proliferation levels in the tumor resection specimens on patient outcome, the results of the Ki67 expression analysis were combined with those of the cleaved caspase-3 expression analysis in a combined apoptosis-proliferation (CAP) parameter (table II). The CAP parameter was not significantly related to TNM stage (p-value 0.211), but was significantly related to the tumor microsatellite status (p-value 0.008). Tumors of both the CAP -+ (Ki67 below median, presence of cleaved caspase-3) and ++ (Ki67 above median, presence of cleaved caspase-3) patients showed significantly more often microsatellite stability compared to the CAP +- (Ki67 above median, absence of cleaved caspase-3) and CAP -- (Ki67 below median, absence of cleaved caspase-3) patients (p-value 0.03). Patients with a CAP ++ tumor showed the best survival outcomes with respect to OS and DFS (figure 3). In the entire cohort patients with a CAP -+ tumor had the worst outcome perspectives. Because tumor microsatellite status was significantly related to the presence of cleaved caspase-3, the next step would be to perform the survival analysis with the CAP parameters stratified for tumor microsatellite status. Unfortunately the number of MSI tumors that was successfully determined was too small to perform this analysis specifically for MSI within this population. We therefore used tumor location, which we previously showed to be highly correlated to tumor microsatellite status, as a surrogate marker in this analysis (figure 4). These Kaplan Meier curves showed in left-sided tumors comparable curves to those presented in figure 3 of the total cohort, but the course of the curves changed in right-sided tumors. The CAP ++ and the CAP -- population within the cohort of left-sided tumors had the best outcome perspectives as opposed to the CAP -+ and +- population that had similar but worse outcome perspectives. The CAP + - actually had, within this left-sided cohort, the worst outcome perspectives (DFS p-value 0.02). In right-sided tumors, the CAP ++ and CAP +- population had the best outcome perspectives as opposed to the CAP - and CAP -+ population that had worse outcome perspectives. We conclude based on these results that combined analysis of apoptosis and proliferation as described with the CAP parameter is related to survival in stage I-IV colon

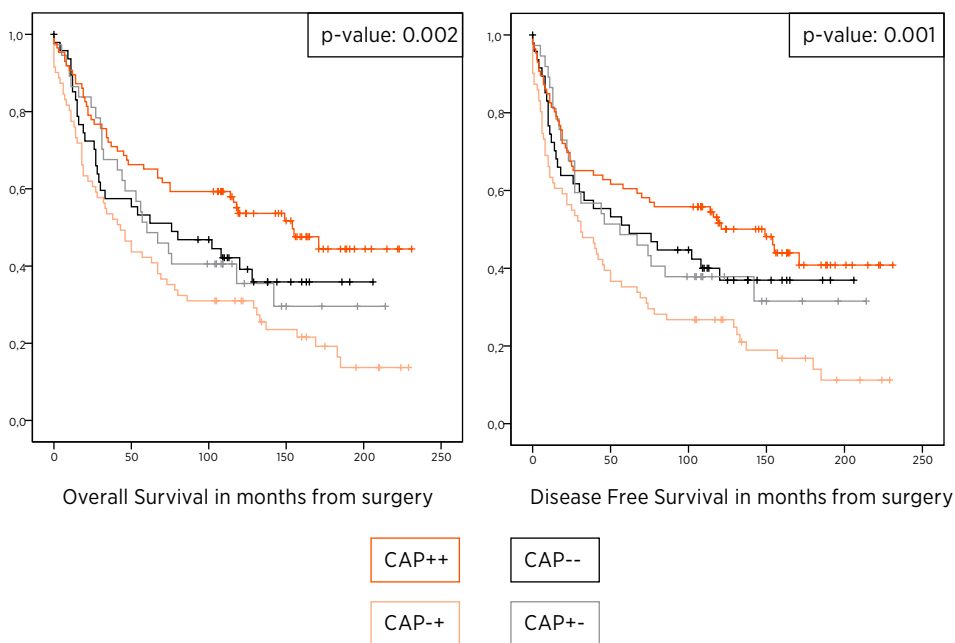


Figure 3

Survival curves stratified for combined tumor apoptosis-proliferation (CAP) expression in colon cancer.

Kaplan Meier curves for OS and DFS in the study population of 285 colon cancer patients stratified for combined tumor apoptosis-proliferation (CAP) expression. This parameter is described in detail in Table II and in the results section.

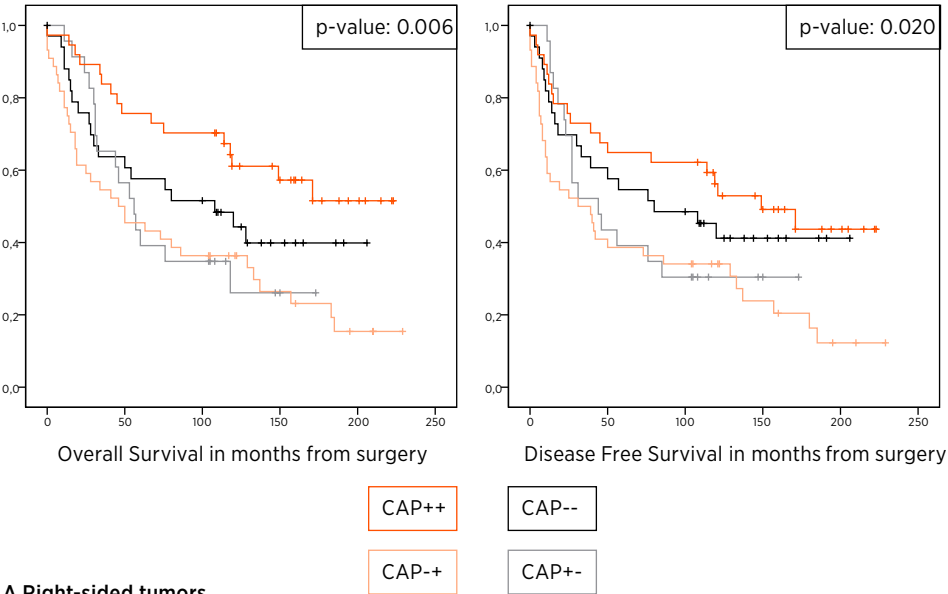
cancer patients. The impact of this parameter on patient outcome, however, varies with tumor location and therefore highly likely with tumor microsatellite status.

Univariate and multivariate analysis

Both for OS and DFS a multivariable analysis was performed including the variables; sex, age at time of operation, TNM stage, tumor grade, administration of adjuvant therapy, microsatellite status, tumor location and the CAP parameter. Age and TNM stage were found to be independent predictors of OS and DFS (Table III & IV). To test whether the effect of the CAP parameter on outcome differed between patients with left- and right-sided tumors, an interaction term was implemented that was borderline significant (p-value 0.06). Although the CAP parameter was not significantly related to TNM stage, stratified analyses for TNM stage showed that the effect of CAP on outcome only remained significant in the stage II patient population. Therefore analysis was again performed with an interaction term, and again this term was borderline significant (p-value 0.05).

Based on these results we conclude that although the CAP parameter is not a statistically independent prognostic indicator of survival in the total patient cohort, the CAP parameter, which is influenced by location, microsatellite stability status and TNM stage, does behold prognostic significance in certain subsets of patients populations such as in stage II, MSS patients.

A Left-sided tumors



A Right-sided tumors

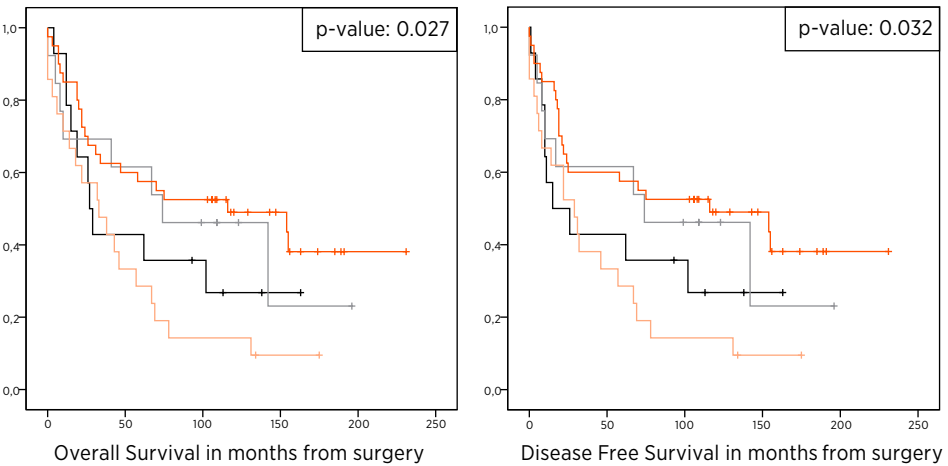


Figure 4

Survival curves stratified for combined tumor apoptosis-proliferation (CAP) expression in colon cancer and stratified for location of the tumor.

(A) Kaplan Meier curves for OS and DFS stratified for the CAP parameter in patients with left sided colon tumors (originating distal to the splenic flexure). (B) Kaplan Meier curves for OS and DFS in patients with right-sided tumors (originating proximal to the splenic flexure). The CAP parameter is described in detail in Table II and in the results section.

Table III

Univariate and multivariable analyses of Overall Survival (OS)

	Univariate analysis			Multivariable analysis		
	HR	95% CI	p-value	HR	95% CI	p-value
TNM			<0.001	1	1.1-3.7	<0.001
1	1			2.01	1.4-4.8	
2	1.57	0.9-2.6		2.61	4.1-14.3	
3	2.17	1.3-3.6		7.67		
4	6.27	3.6-10.7				
Age	1.04	1.026-1.053	<0.0001	1.054	1.037-1.071	<0.001
CAP			0.006	1	0.6-1.9	0.28
--	1.00			1.10	0.8-2.0	
+-	1.42	0.9-2.4		1.27	0.5-1.4	
+-	0.71	0.5-1.0		0.85		
++	0.52	0.3-0.9				

This table provides the data for the univariate en multivariable analysis of OS. The univariate analysis included sex, age, tumor grade, adjuvant therapy administration, microsatellite status, TNM stage, the CAP parameter, microsatellite status and tumor location. The CAP parameter, age and TNM stage were all significant predictors of OS in univariate analysis. In multivariable analysis only TNM stage and the patient age at time of surgery retained significance. The CAP parameter is therefore not an independent predictor of overall survival in stage I-IV in this cohort of colon cancer patients.

Table IV

Univariate and multivariable analyses of Disease Free Survival (DFS)

	Univariate analysis			Multivariate analysis		
	HR	95% CI	p-value	HR	95% CI	p-value
TNM			<0.001			<0.001
1	1			1		
2	1.56	0.9-2.5		1.94	1.1-3.5	
3	2.25	1.4-3.7		2.52	1.4-4.6	
4	6.14	3.6-10.4		7.17	3.9-13.1	
Age	1.032	1.019-1.045	<0.001	1.043	1.028-1.059	<0.001
CAP			0.02			0.235
--	1			1		
+-	1.04	0.6-1.8		1.08	0.6-1.8	
+-	1.60	1.0-2.5		1.37	0.9-2.1	
++	0.75	0.5-1.2		0.91	0.6-1.5	

This table provides the data for the univariate en multivariable analysis of DFS The univariate analysis included sex, age, tumor grade, adjuvant therapy administration, microsatellite status, TNM stage, the CAP parameter, microsatellite status and tumor location. The CAP parameter, age and TNM stage were all significant predictors of DFS in univariate analysis. In multivariable analysis only TNM stage and the patient age at time of surgery retained significance. The CAP parameter is therefore not an independent predictor of disease free survival in this cohort of stage I-IV colon cancer patients.

DISCUSSION

Our study shows that a combined parameter, CAP, describing the level of tumor cell proliferation and apoptosis is significantly related to patient outcome in a stage I-IV colon cancer patient cohort with respect to DFS and OS. Although counter intuitively, patients with CAP ++ tumor, showing high levels of both proliferation and apoptosis, showed the best clinical outcome perspectives. The effect of the CAP parameter, however, varied with TNM stage and tumor location and was significantly related to tumor location and tumor microsatellite status. These results confirm our hypothesis that clinical outcome is dependent on both tumor cell proliferation and apoptosis.

The processes of both tumor cell proliferation and apoptosis both have been extensively studied with varying results in many types of cancer. In general, high tumor cell proliferation levels were associated with aggressive tumor development and progression (21;25). However, other studies reported on an inverse association between tumor cell proliferation level and clinical outcome (8;18;23;26). These latter results are in line with what we have found: a better outcome perspective in colon cancer patients with high levels of tumor cell proliferation. In this study we were not able to establish a relationship between the level of apoptosis as a single marker and patient outcome in colon cancer patients. Although there are studies that describe a link between tumor cell apoptosis and clinical outcome, for example Jonges *et al.* who described cleaved caspase-3 expression as a prognostic marker in colon cancer patients (20), the majority of the studies have presented us with more ambiguous results (1;16;17;27;28).

The contradicting results derived from studies reporting on either proliferation or apoptosis in colon cancer strengthened our hypothesis that a balance between both these processes determines patient's clinical outcome. Michael-Robinson *et al.* previously reported on a cohort of 100 colorectal cancer patients in which they determined an Apoptotic Index: Proliferation Index (AI:PI) ratio (26). This AI:PI ratio was based on M30 IHC for the apoptosis level and Ki67 IHC for the proliferation level. They were able to determine a relationship between the proliferation index and outcome comparable to our results: they also related their AI:PI index significantly to patient outcome. In previous studies the use of the apoptotic index has been criticized as researches found the use of the parameter to be accompanied with high amounts of interobserver variability (29). Therefore we didn't use a continuous variable based on counted percentages, but developed a more descriptive parameter, the CAP, to determine the combined effect of apoptosis and proliferation within our patients tumor samples. The differences in outcome parameters and also patient selection make it difficult to perform a one-to-one correlation of the results of Michael-Robinson *et al.* and our results. Their conclusions though do affirm our hypothesis. Interestingly, the survival difference they found between a high and low AI:PI index was similar in both MSS and MSI patients. Our results showed that the effect of the CAP parameter differed between tumors emerging from colon proximal and colon distal to the splenic flexure. In the left-sided cohort

the patients with CAP -- and ++ tumors performed better with respect to outcome. In the right-sided cohort the CAP +- performed significantly better than the CAP -- cohort. This is comparable to what we have previously found and described by Jonges *et al.* (20). The effect of apoptosis on patient outcome is related to tumor location. Based on our results we hypothesize that it is either tumor microsatellite status as suggested by both Jonges and Michael-Robison, or tumor location which might influence the balance between tumor cell proliferation and apoptosis and therefore patient outcome (20;26). The concept of the effect of tumor location is in accordance with what has recently been described by The Cancer Genome Atlas Network in their publication in Nature in 2012, who tested the hypothesis that differences between tumors originating from the left or the right side of the colon is not based on their microsatellite status but it might be caused by the different embryonic origins of the right- and left-sided colon (30).

It is not unlikely that the tumor microsatellite status influences the balance between tumor cell proliferation and apoptosis. Microsatellite instable tumors are known to have high levels of proliferation and tend to accumulate gene mutations leading to increased production of abnormal peptides (31;32). This phenotype has been hypothesized to cause an immune reaction resulting in higher levels of apoptosis, eventually resulting in better patient outcome (33).

In conclusion, the CAP variable described in this study reflects the balance between the apoptosis and proliferation in colon cancer tissue and showed to be related to patient outcome. These results confirm our hypothesis that apoptosis and proliferation together determine patient outcome in colon cancer and this relation is influenced by tumor location and/ or by tumor microsatellite instability. This was shown by the different effects of the CAP parameter on patient outcome in the left and right-sided colon cancer patients cohorts and the statically significant relation of the level of apoptosis with tumor microsatellite status, also described in previous studies (20,26). Important steps have been taken towards the implementation of a CAP like parameter into clinical practice, such as the development of the CDK1 SA (Cyclin Dependent Kinase 1 Specific Activity Assay), a biochemical assay that can replace the Ki67 IHC, and the improvement of the existing biochemical assays to measure cleaved caspase-3 activity for easy clinical use (24). Further studies should focus on the design of clinical tests combining both proliferation-based markers and apoptosis-based markers into one analysis to assure clinical applicability.

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CHAPTER 4

Specific activity of cyclin-dependent kinase I is a new potential predictor of tumor recurrence in stage II colon cancer

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British Journal of Cancer. 2012; 106 133-140

ABSTRACT

Background: There are no established biomarkers to identify tumor recurrence in stage II colon cancer. As shown previously, the enzymatic activity of the cyclin dependent kinases 1 and 2 (CDK1 and CDK2) predicts outcome in breast cancer. Therefore, we investigated whether CDK activity identifies tumor recurrence in colon cancer.

Methods: In all, 254 patients with completely resected (R0) UICC stage II colon cancer were analyzed retrospectively from two independent cohorts from Munich (Germany), and Leiden (Netherlands). None of the patients received adjuvant treatment. Development of distant metastasis was observed in 27 patients (median follow-up: 86 months). Protein expression and activity of CDKs were measured on fresh-frozen tumor samples.

Results: Specific activity of CDK1 (CDK1SA), but not CDK2, significantly predicted distant metastasis (concordance index = 0.69, 95%CI: 0.55–0.79, $p=0.036$). Cut-off derivation by maximum log-rank statistics yielded a threshold of CDK1SA at 11 (specific activity units, $p=0.029$). Accordingly, 59% of patients were classified as high-risk (CDK1SA ≥ 11). Cox proportional hazard analysis revealed CDK1SA as independent prognostic variable (hazard ratio = 6.2, 95% CI: 1.44–26.9, $p=0.012$). Moreover, CDK1SA was significantly elevated in microsatellite stable tumors.

Conclusion: Specific activity of CDK1 is a promising biomarker for metastasis risk in stage II colon cancer.

INTRODUCTION

Each year >1 million individuals worldwide develop colon cancer with a disease specific mortality rate of almost 33% (1-3). Approximately 40% of resected colon cancers are from stage II (T3-4N0M0). The 5 year survival rates vary between 88% in T3N0 patients, and 75% in T4N0 patients. Chemotherapy is widely accepted as adjuvant treatment for stage III patients, whose 5 year survival (stage III A and B) is higher than 75% (4). Use of chemotherapy for stage II, T4 patients remains controversial despite their worse survival rates. This indicates that the allocation of treatment based solely on conventional staging methods is not optimal (5-9). Over the last decade there have been important developments towards the discovery of new prognostic and predictive markers that might improve staging methods. The American Society of Clinical Oncology's Tumor Markers Expert Panel (ASCO TEMP-2006) and its European counterpart, the European Group on Tumor Markers (EGTM-2007) have recently reviewed the literature on these biomarkers. However, all biomarkers reviewed lacked the significant, discriminative value that is required to become implemented into clinical practice (10-12). There is a stringent need for new assays that are able to identify stage II colon cancer patients who might benefit from adjuvant therapy. Genomic instability and altered cell proliferation are major contributors to tumor growth and aggressiveness. Measuring these hallmarks of colon cancer in a quantitative fashion could be a suitable option for risk stratification. The proliferation rate of tumor cells has so far been studied with methods such as ³H-thymidine/BrdU incorporation, mitotic index, or Ki-67/PCNA immunohistochemistry, but none of these tests have reached clinical application (13;14). Therefore, analysis of the highly conserved drivers of the cell cycle, the cyclin-dependent kinases (CDKs) 1 and 2, may be a more promising approach (15). CDK expression is constitutive in tumors but their enzymatic activity changes markedly according to the specific cell cycle phase. On the molecular level, the activity of CDK is regulated by subunits known as cyclins, and by phosphorylation of conserved tyrosine and threonine residues. Over-expression of cyclins, as well as inactivation of CDK inhibitors, are well documented as prognostic markers for esophageal, gastric, colorectal, breast and lung cancer (16-22). However, expression analysis of cyclins and other factors may not necessarily indicate the enzymatic activity of CDKs, which is crucial for the cell cycle status of the cancer cells. We have recently reported an assay that measures the specific activity of CDK 1 and CDK2 (23-25), based on a well standardized biochemical assay that requires only small amounts of fresh frozen tissue and is (23). The hallmark of this approach is the extraction of functional CDK enzyme from tumor tissue, followed by determination of its kinase activity. We hypothesize that intratumoral kinase activity of CDKs predicts the prognosis of tumor patients with great fidelity, because it directly represents a quantifiable readout for two hallmarks of tumors: increased proliferation and genomic instability. Two large, independent cohorts of breast cancer patients demonstrated that this assay had prognostic value (24;25). A CDK-based risk score validated in these studies was a significant and independent prognostic factor, especially for distant

recurrence. The aim of this study was to determine the ability of CDK-based analysis to predict recurrence in patients with locally restricted colon cancer. The study was carried out retrospectively on two independent patient cohorts derived from large surgical oncology centers in the Netherlands and Germany. Our results demonstrate that the specific activity of CDK1 identifies stage II colon cancer patients with a high risk of distant disease recurrence. This patient group may benefit from adjuvant chemotherapy, which would not be recommended according to standard criteria.

MATERIALS & METHODS

Patients

The study was approved by the local ethics committees at LUMC and TUM. Informed, written consent had been obtained prior to the study. Fresh frozen samples of 271 of stage II colon carcinomas were analyzed, collected at Leiden University Medical Center (LUMC, 1985 - 2005), and at Klinikum rechts der Isar (TUM, 1987 to 2006). All patients had curative (R0) tumor resection, and none of them received adjuvant or neoadjuvant therapy. Tumor tissue was dissected immediately after resection by a pathologist, snap frozen in liquid nitrogen and stored at -80°C . Development of distant metastasis was observed in 27 patients (11%) after a follow-up of 7.2 years (median). Five samples (1.8%) were excluded due to tumor cell content of less than 10 percent. All remaining tissue samples underwent C2P-analysis, 12 cases were excluded due to assay failure, or CDK expression level below detection threshold ($n=3$). Of note, all 12 excluded cases were free of tumor recurrence. Hence, 254 samples were available for further analysis ($n=217$ from TUM, and $n=37$ from LUMC).

Determination of CDK-specific activities

Ten to 20 sections of $100\text{ }\mu\text{m}$ thickness were cut with a cryostat and subjected to CDK analysis. One section of $7\text{ }\mu\text{m}$ thickness was cut from the middle of each block and evaluated by a pathologist after standard H&E staining. Cases with tumor cell content $<10\%$ were excluded. The system to measure the CDK specific activity (CDKSA) is called "C2P" (for "Cell Cycle Profiling"; Sysmex, Kobe, Japan; Ishihara *et al*, 2005; Kim *et al*, 2008). In brief, lysates of frozen material were applied to a well of 96-well PVDF filter plate (Millipore, MA, USA). Expression of CDKs was detected quantitatively by sequential reactions with primary anti-CDK antibodies, biotinylated anti-rabbit antibodies, and fluorescein-labeled streptavidin. To measure the kinase activity, CDK molecules were immunoprecipitated from the lysate using protein beads, as reported in detail earlier (Ishihara *et al*, 2005; Kim *et al*, 2008). CDKSA was calculated as CDK kinase activity units (aU/ μL lysate) divided by its corresponding CDK expression units (eU/ μL lysate). Both, aU (CDK activity unit), and eU (CDK expression unit) were defined as the expression and activity equivalent to 1 ng of recombinant CDK1, and CDK2, respectively. The distribution

of the CDK1SA and CDK2SA within the LUMC and the TUM cohort can be found in supplementary Figure 1. Further details regarding the quality controls for this assay can be found in the supplementary data.

Immunofluorescence analysis

Tissue specimens (7 µm) from 207 samples were available for evaluation by immunofluorescence microscopy (Axiovert 200, Zeiss, Göttingen, Germany). After fixation with 3% PFA and antigen retrieval (10 min boiling, sodium citrate buffer, pH=6.0), slides were incubated with anti-Ki-67 antibody (clone MIB-1, M 7240, DAKO) and/or anti-cytokeratin-20 antibody (rabbit monoclonal, 2039-1, Epitomics, Burlingame, CA) diluted 1:200, followed by incubation with secondary antibodies (Molecular Probes, Darmstadt, Germany; Dianova, Hamburg, Germany), and counterstaining with 4', 6'-diamidino-2-phenylindole (DAPI, Invitrogen, Darmstadt, Germany). Ki-67 positive nuclei from CK20 positive cells were regarded as *bona fide* tumor cells and were counted in a semi-automated manner using ImageJ freeware (<http://rsb.info.nih.gov/ij/>).

MSI (Microsatellite instability) determination

Tissue from 200 patients of the Munich cohort and all 37 patients of the LUMC was available for DNA isolation with the QIAampDNA™Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA concentration and quality was checked with an ND-1000 NanoDrop Spectrophotometer (Thermo Fisher, Schwerte, Germany). Subsequently, microsatellite instability was tested with the MSI Analysis System, Version 1.2 (Promega, Mannheim, Germany). This assay co-amplifies five mononucleotide repeat markers; BAT-25, BAT-26, NR-21, NR-24, and MONO-27 to determine MSI status. It includes two pentanucleotide repeats, Penta C and D, to make sure that normal and tumor samples are derived from the same patient. The results of this assay have been previously compared with the Bethesda panel markers and proven highly sensitive for MSI determination (26). The MSI status was determined for 32 of the 37 LUMC cases, and for 191 of 200 TUM patients. In 6% of the cases (14 of 237 available DNA samples), MSI status could not be determined based on evaluation of the PCR array data by an experienced pathologist due to ambiguous results.

BRAF

The mutational status of the oncogene BRAF (V600E, GTG>GAG substitution in exon 15) was assessed by high resolution melting analysis of genomic DNA on a Lightcycler 480 II platform (Roche, Mannheim; SYBR Green I /HRM Dye Protocol), in accordance to published protocols (23). Briefly, 20 ng of genomic DNA (10 ng/µL) were amplified in total volume of 20 µL with 10 µL High Resolution Master Mix, 2 mM MgCl₂, and 1 mM each of oligonucleotide primers, 2µL template DNA and 4.8 µL dH₂O. Primer sequences were: BRAF Exon 15 For: 5'-TGA AGA CCT CAC AGT AAA AAT AGG-3', BRAF Exon 15 Rev: 5'-TCC AGA CAA CTG TTC AAA CTG AT-3'. After pre-incubation (95°C, 10 min), amplification of a 147 Bp product was carried

out in 40 cycles (95°C, 15 sec / 61°C, 15 sec / 72°C, 15 sec.), followed by melting point analysis with an initial phase: 95°C, 5 sec, and 72 °C, 90 sec, followed by a melting profile ranging from 72°C to 95°C in 25 min. As positive control, genomic DNA from the BRAF-mutated colon cancer cell line HT29 was used.

Statistical analysis

Statistical analyses were conducted using R Software version 2.11.1 (R Foundation for Statistical Computing, Vienna, Austria). In order to derive optimal cut off values of quantitative CDK measurements for recurrence risk stratification, maximally selected log-rank statistics have been used. To consider multiple test issue within these analyses, the R-function 'maxstat.test' was employed (27). To internally validate the derived cut-off, the entire data set was randomly divided in a training and test set (ratio: 70:30). Furthermore, bootstrap re-sampling analysis was conducted to estimate distribution of derived cut-off values and 95% confidence intervals respectively. Multivariable Cox-regression was performed to assess recurrence risk differences between derived sub-groups in simultaneous consideration of potential confounding factors. Due to the low number of critical events, multivariable regression analyses had to be performed consecutively (one by one inclusion of potential confounding factors) to avoid over-adjustment. By the use of survival-ROC analysis, predictive capability of recurrence risk stratification was assessed cumulatively over the course of the follow-up. In this term, area under the time dependent ROC-curve (concordance index) was reported with 95% bootstrap confidence interval. The Kaplan-Meier Methods was used for survival plotting and log-rank test for comparison of survival curves. All statistical tests were conducted two-sided and a p-value <0.05 was considered significant.

RESULTS

We have determined the specific activity of CDK1 and CDK2 (CDK1SA and CDK2SA) in a study population comprised of samples from two independent cohorts of stage II colon cancer patients originating from the Leiden University Medical Centre (LUMC, The Netherlands) and the Klinikum Rechts der Isar, of the Technical University in Munich (TUM, Germany). Five samples (1.8%) were excluded due to tumor cell content of less than 10 percent. Twelve cases were excluded due to assay failure, and in three cases the CDK expression levels were below the detection threshold. Of note, all excluded cases were free of tumor recurrence. Altogether, the expression and kinase assay ("C2P", in short for "Cell cycle profiling") yielded results in 96% of patients (254 out of 266; n=217 from TUM, and n=37 from LUMC). There were no statistically significant differences in clinico-pathological characteristics between both cohorts (table 1). The specific activity (SA) was calculated and indicated as kinase activity in relation to its corresponding mass concentration. The CDK activity unit and CDK expression unit were defined as the equivalent of 1 ng recombinant CDK protein. The distribution of the CDK1SA did not vary significantly

Table 1

patient characteristics

Category	Subcategory	Total collec- tive(%)	Patients from TUM (%)	Patients from LUMC (%)
Total		254 (100%)	217 (100%)	37 (100%)
Sex	Male	141 (56)	124 (57)	17 (46)
	Female	113 (44)	93(43)	20 (54)
Age		65 (median)	65 (median)	69 (median)
		15-91 (range)	15-91 (range)	26-82 (range)
Open surgery		254 (100)	217 (100)	37 (100)
Location	Caecum	40(16)	31 (14)	9 (24)
	Ascending colon	66 (26)	59 (27)	7 (19)
	Transverse colon	26 (10)	23 (11)	3 (8)
	Descending colon	30 (12)	28 (13)	2 (5)
	Sigmoid	92 (36)	76 (35)	16 (43)
Tumor size		6 (median)	6 (median)	5 (median)
		2-15 (range)	2-15 (range)	3-14 (range)
PT	T3	221 (87)	188 (87)	33 (89)
	T4	33 (13)	29 (13)	4 (11)
Lymph nodes total		19 (median)	20 (median)	10 (median)
		1-72 (range)	7-72 (range)	1-26 (range)
Grading	G1,G2	170 (67)	149 (69)	21 (57)
	G3,G4	77 (30)	65 (30)	12 (32)
	Missing	7 (3)	3 (1)	4 (11)
Recurrence	None	220 (87)	191 (88)	29 (78)
	Distant	27 (11)	22 (10)	5 (14)
	Local	7 (3)	4 (2)	3 (8)
Survival information	Alive	172 (68)	155 (71)	17 (46)
	Tumor-related death	25 (10)	19 (9)	6 (16)
	Non-tumorrelated death	58 (23)	44 (20)	14 (38)

Abbreviation: pT= tumour stage

between the two study cohorts (Mann-Whitney U test, $p=0.35$), whereas the average of CDK2SA was higher in samples from the Netherlands ($p=0.012$).

Predictive performance and cut-off derivation of CDK specific activity for distant recurrence

The distribution of clinical samples was plotted on a scatter diagram according to CDK1SA and CDK2SA (figure 1A). Cases with distant metastasis clustered in the region with high CDK1 activity, suggesting that mainly CDK1SA could have prognostic power. In order to evaluate the prognostic performance of CDK activity for distant metastasis risk, the true positive rates of distant disease recurrence (sensitivity) and corresponding false positive rates (100-specificity) were summarized in a time-dependent receiver operating characteristic (ROC) curve. The average area under the ROC curve (concordance index or AUC) was 0.69 for CDK1SA (95%CI:

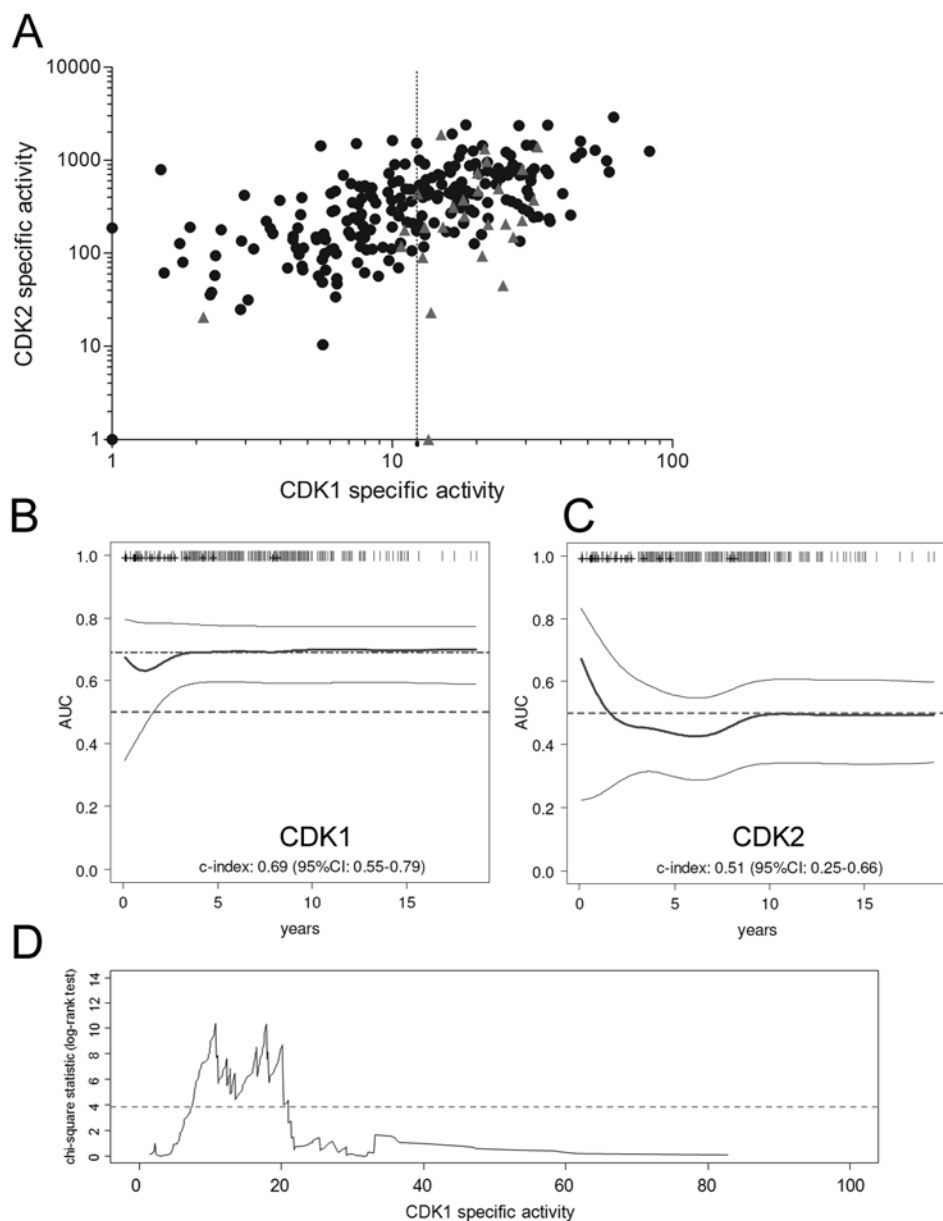


Figure 1

Prognostic performance of the specific activities of CDK1 and CDK2. (A) All cases (n=254) spotted on a scatter diagram with logarithmic scales according to CDK1 SA and CDK2 SA, respectively. Grey triangle: patient with distant metastasis, black dot: no metastasis. (B and C) Time-dependent ROC analysis against CDK1 SA (B) or CDK2 SA (C). Thick line: concordance index, thin line: 95% CI. Concordance index was 0.69 for CDK1 SA (95% CI: 0.55-0.79, $P=0.036$), and 0.51 for CDK2 SA (95% CI: 0.25-0.66, $P=0.57$). (D) Derivation of an optimal CDK1 SA cutoff value. The maximum log-rank test statistic was obtained when CDK1 SA was 11 or 18 (maUeU⁻¹).

0.55 to 0.79, $p=0.024$), and 0.51 for CDK2SA, respectively (95%CI: 0.29 to 0.66; $p=0.57$) (figure 1B, C). Combined, these results suggested that CDK1SA, but not CDK2SA, is valuable for long-term distant recurrence prediction. Therefore, we focused on CDK1SA and derived the statistically best discriminating cut-off value for CDK1SA, as indicated by maximum log-rank test. For 254 cases, two local maxima of log-rank test statistic were obtained, one for CDK1SA=11 (milli-activity unit per expression unit, maU/eU), and one for CDK1SA=18 (maU/eU) (figure 1D). In order to test the robustness of the selected cut-off values, a second cut-off derivation was performed using the subset of samples with CDK1SA>11 (maU/eU) ($n=150$). In this analysis, the previously proposed cut-off value of 18 (maU/eU) neither showed a significant maximum peak, nor was considerably elevated compared to the other candidate cut-off values. This result suggested that the optimal cut-off value for CDK1SA was indeed at 11 (maU/eU). The final bootstrap analysis confirmed a cut-off value for CDK1 SA of 11 (maU/eU) to be of sufficient discriminant value for further analysis. In conclusion, patients with CDK1 activity level > 11 (maU/eU) were classified in the high-risk group ($n=104$, 40% of the patients), and the remaining patients as low-risk ($n=150$, 60%).

CDK1-based risk prediction for distant metastasis-free survival and cause-specific survival

Univariable “time to event” analysis showed that patients from the CDK1SA-based low risk group had significantly longer distant metastasis-free intervals than patients in the high risk group (HR = 6.2; 95%CI: 1.45 to 26.9; $p=0.0049$) (figure 2A). Importantly, this finding was retained to be statistically significant after adjusting for the multiple log-rank testing which had been performed in order to obtain the optimal cut-off value of 11 (maU/eU) (exact conditional Monte-Carlo p -value = 0.029). The independence of prognostic ability of CDK1SA-based recurrence risk stratification was further evaluated and finally confirmed by multivariable analyses (table 2). Hazard ratio estimates remained nearly unchanged after consecutive adjustment for the most important clinical-pathological variables which are currently used for risk evaluation in stage II colon cancer: T4, poor differentiation, presence of obstruction or perforation, lymphatic and vessel invasion, high CEA level, and ≤ 12 regional lymph nodes examined (24;25)(table 2). Next, a putative confounding influence of mutations in the BRAF oncogene were analyzed. In 217 patients, tissue was available for high resolution melting analysis of mutations in exon 15 of BRAF. In 32 cases (14.8%), BRAF^{V600E} mutations were detected, 183 patients had BRAF wild-type status, and 2 cases were not informative. In Kaplan-Meier analysis, the BRAF mutation status was not significantly associated with metastasis-free survival ($p=0.337$), nor with cause-specific survival ($p=0.253$; not shown), and it was excluded as confounding factor for CDK1SA-based risk prediction (table 2). However, when considering stroma content as adjustment variable, a lack of statistical significance was apparent for the effect of dichotomized CDK1SA. The apparent absence of significance may be explained by the reduced statistical power for this parameter, since about 30% of the cases lacked available stroma content

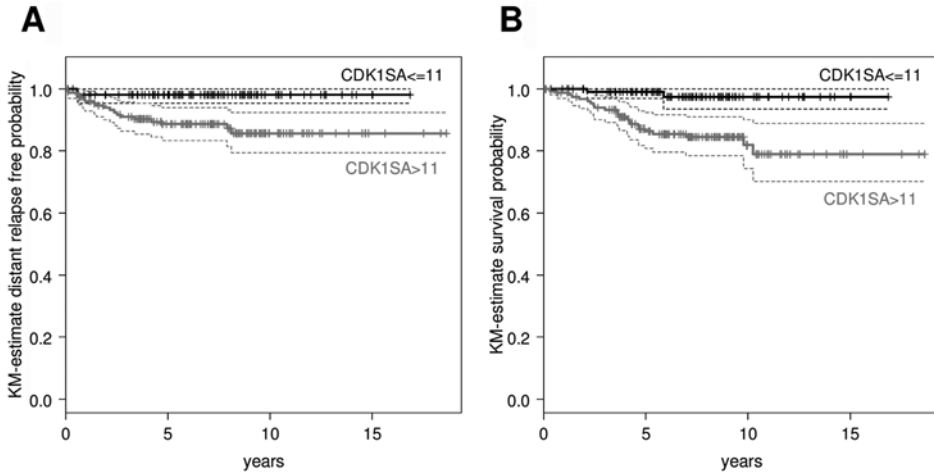


Figure 2

Analysis of distant metastasis-free survival and cause-specific survival. (A) Patients classified in the high-risk group (based on $CDK1SA > 11 \text{ maUeU}^{-1}$) had a significantly worse distant metastasis event rate as compared with the low-risk group (HR= 6.2, 95% CI: 1.45-26.9, $P = 0.0049$; exact conditional Monte-Carlo P -value = 0.029). (B) Patients classified in the CDK1 SA-based high-risk group had a significantly lower cause-specific survival (HR=7.62, 95% CI: 1.80-32.2, $P = 0.001$).

data. Twenty-five patients (10%) died during the follow-up, among them were all 20 patients with distant metastases, and only five patients with no evidence for distant metastases, but with local tumor recurrence. Due to this strong association of distant relapse and death, CDK1SA categorization was found to be a significant predictor for cause-specific survival (HR high-risk vs. low-risk group: 7.62; 95%CI: 1.80-32.2; $p=0.001$) (figure 2B). This result was thoroughly confirmed in the multivariable analyses. All adjusted estimates of the hazard ratio showed values of >7.75 , with lower 95% confidence limits >1.80 , and p -values <0.01 . However, a non-significant hazard ratio was estimated after adjustment for stroma content (HR high-risk vs. low-risk group: 5.22; 95%CI: 0.65-41.5; $p=0.12$).

Correlation between CDK specific activity, cell proliferation and microsatellite status

Based on the knowledge of the process of tumorigenesis, high CDK1SA levels could be a reflection of strongly elevated tumor cell proliferation rates. Therefore, we have analyzed tumor cell proliferation with the established proliferation marker Ki-67. The Ki-67 labeling index, defined as the percentage of Cytokeratin20-positive cancer cells with Ki-67-positive nuclei, was determined for $n=207$ cases. The median of the Ki-67 index was 21.4%, but it was not retained by Cox regression analysis as significant prognostic factor for distant metastasis (HR = 0.69; 95%CI: 0.02 to 24.0; $p=0.84$). Next, a putative correlation between CDK1SA and the Ki-67 index was examined. However, no significant correlation was found between CDK1SA and Ki-67 index (Spearman's $\rho=+0.04$; $p=0.54$) (figure 3).

Table 2

Consecutive (one-by-one) adjustment for confounding factors.

Pairwise comparison	Category	Subcategory	HR	95% CI for HR Lower	Upper	P-value
1	CDK1 SA	>11 vs ≤11	4.23	0.52	34.11	0.180a
	Stroma content (%)		1.02	0.99	1.04	0.230
2	CDK1SA	>11 vs ≤11	6.24	1.44	26.93	0.014
	Histol. grade	>2 vs ≤ 2	0.99	0.13	7.49	>0.99
3	CDK1 SA	>11 vs ≤11	6.29	1.46	27.10	0.014
	pT stage	4 vs 3	1.29	0.38	4.40	0.69
4	CDK1 SA	>11 vs ≤11	6.52	1.51	28.14	0.012
	Sex	Female/male	0.48	0.18	1.25	0.130
5	CDK1 SA	>11 vs ≤11	6.57	1.51	28.54	0.012
	Age	(years)	1.01	0.98	1.05	0.470
6	CDK1 SA	>11 vs ≤11	5.6	1.287	24.42	0.022
	LN resected	>12 vs ≤ 12	0.52	0.21	1.31	0.165
7	CDK1 SA	>11 vs ≤11	8.23	1.07	63.54	0.043
	CEA	(serumlevel)	0.99	0.89	1.10	0.828
8	CDK1 SA	>11 vs ≤11	11.08	1.46	83.93	0.020
	Obstruction	Yes/No	0.68	0.19	2.39	0.545
9	CDK1 SA	>11 vs ≤11	9.85	1.29	75.32	0.028
	Perforation	Yes/No	0.00	0.00		0.988
10	CDK1 SA	>11 vs ≤11	11.43	1.51	86.73	0.018
	Lymphinvasion	Yes/No	0.22	0.63	7.73	0.219
11	CDK1 SA	>11 vs ≤11	11.44	1.51	86.73	0.018
	Angioinvasion	Yes/No	4.75	0.62	36.69	0.135
12	CDK1 SA	>11 vs ≤11	11/17	1.48	84.57	0.019
	BRAF	Mutated/WT	0.39	0.05	2.92	0.356

Abbreviations: CI = confidence interval; HR = hazard ratio; pT = tumour stage; WT = wild type. ^a82 cases (32%) with missing value for stroma content. CDK1SA is not significant (P = 0.180), however; in all other test against confounding factors, CDK1 SA achieved significance.

Lastly, a putative correlation between genomic instability and CDK1 activity was tested, since CDKs have been shown to be implicated in cellular responses to genetic instability. Microsatellite instability (MSI), caused by defects in the cellular mismatch repair system, has been suggested for colorectal cancer as a favorable prognostic marker. The microsatellite instability status was determined with standard methods for 223 cases, and a high level of instability was detected in 59 tumors (26.5%, MSI-High), whereas 164 samples showed stable microsatellite repeats (73%, MSS). Cox regression analysis indicated an estimated five-fold risk-difference regarding distant metastasis free survival for microsatellite stable patients, but the results did not attain significance (HR 5.898; CI95% 0.782-44.481; p=0.085). A significant association of microsatellite instability and CDK1SA-based risk stratification was apparent, based on the cut-off for CDK1SA of 11 (maU/eU). In the patient group with stable microsatellites, significantly more cases with

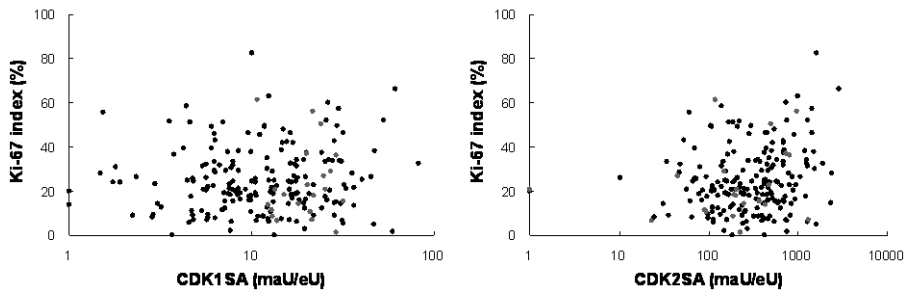


Figure 3

Correlation between CDK SAs and Ki-67 index (percent of Ki-67 positive cells of all CDK20-positive tumour cells). Cases were plotted on a scatter diagram according to Ki-67 index against CDK1 SA (left), or CDK2 SA (right). Grey circle: tumor with distant metastasis. Ki-67 showed a weak but significant positive correlation with CDK2 SA (Spearman's $\rho = 0.17$, $P = 0.016$), but not with CD1 SA (Spearman's $\rho = 0.54$).

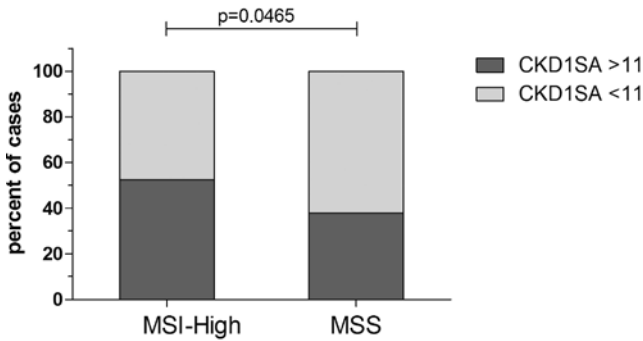


Figure 4

Association of CDK1 SA-based risk stratification with microsatellite-stable phenotype. Among the patients with a stable microsatellite phenotype (MSS), 62% (102 out of 164) were classified in the high-risk group based on CDK1 SA. On the other hand, 47.5% (28 out of 59) of the patients with high MSI (MSI-H) were classified as high-risk, based on the CDK1 SA threshold (χ^2 -test, $P = 0.0465$).

elevated CDK1 specific activity were observed (chi-square test: $p = 0.0465$, figure 4). However, a direct comparison of CD1SA between patients with stable or unstable microsatellites did not attain significance.

DISCUSSION

This study is the first report demonstrating the specific activity of CDK1 (CDK1SA) as prognostic biomarker for stage II colon cancer in a blinded and retrospective manner. Two patient cohorts from Germany and the Netherlands were included in this study. Essentially, no differences were observed between these cohorts regarding clinical parameters or CDK1 activity, indicating that the patients were recruited in an unbiased manner. However, the average of CDK2SA was slightly but significantly higher in the samples from the Netherlands. This may be due to differences in sample embedding and preparation between the study centers, and to technical variations between the assay systems for CDK1SA and CDK2SA. Previously, CDK1SA- and CDK2SA-based risk was shown to be a clinically useful prognostic marker of early breast cancer of Caucasian and Asian cohorts (23-25).

To identify patients with unfavorable prognosis who might benefit from adjuvant chemotherapy, several types of staging systems have been developed (4;7;28;29). The current staging systems, however, do not provide accurate risk assessment for stage II patients (29). Moreover, a number of molecular markers have been proposed, such as mutations in KRAS and TP53, loss of heterozygosity of chromosome 18, and microsatellite instability (12;30;31). However, none of these candidate biomarkers has yet clearly proven to be useful for diagnosis or staging of patients with stage II colorectal cancer, except for mutations in the BRAF oncogene, which were found to be prognostic for overall survival, particularly in patients with microsatellite stable tumors (32;33). Comprehensive approaches using “omics” technologies have been applied to find biomarkers for colorectal cancer, and we and many others have proposed prognostic transcriptome profile sets so far (34-38). However, inter-patient and even intra-tumoral heterogeneity, as well as cost factors have precluded wide-scale clinical application. A promising strategy to circumvent tumor heterogeneity is to focus on the central hallmarks of cancer, which are present in almost all tumors irrespective of the underlying molecular changes. Altered cell proliferation and genomic instability are central hallmarks in the case of colon cancer (15;39). Therefore, we focused on the enzymatic activities and protein expression of cyclin dependent kinases (CDKs), the main drivers of cell cycle progression. Moreover, CDK regulators have been well documented as prognostic indicators in many solid tumors (16-22).

Indeed, CDK1SA was a substantial and constant marker for long-term event prediction of distant metastasis in the present study. A robust cut-off value for CDK1SA was derived by choosing a threshold with maximum log-rank statistics (27). Importantly, the cut-off value of 11 (maU/eU) was verified by the adjusted multiple log-rank test. Multivariate analysis retained CDK1 specific activity as independent predictor of distant recurrence. None of the currently accepted clinical risk factors, e.g., T4 stage, poor differentiation, obstruction or tumor perforation (40), was identified as confounding factor (table 2). Moreover, CDK1SA was independent of the mutation status in the BRAF oncogene. Therefore, we conclude that CDK1SA-based risk stratification is a reliable prognostic marker for distant metastasis in

stage II colon cancer. Two hypotheses, which are not mutually exclusive, may explain the increased intratumoral CDK1SA level in patients with worse prognosis. First, specific activity of CDK1 may directly reflect higher cancer cell proliferation. To address this question, we have examined a putative correlation between CDK1SA and proliferation. The index of proliferating cancer cells did not significantly correlate with CDK1SA. Moreover, the Ki-67 proliferation index itself was not significant for prognosis, in accordance with earlier findings (41). Second, CDK1 activity may be elevated due to chromosomal instability, a factor already associated with worse prognosis (40). Indeed, high CDK1SA levels were significantly correlated with a stable microsatellite phenotype (chi-square test: $p = 0.0465$). To the best of our knowledge, no reports exist that provide a cause-and-effect link between CDK1 activity and microsatellite instability. However, colorectal tumors with stable microsatellites are thought to present chromosomal instability (CIN), associated with worse prognosis. Thus, microsatellite-stable tumors with high CDK1SA levels in our collective are likely to display chromosomal instability. On the molecular level, regulation of CDK1 activity is orchestrated by cellular checkpoints. Altered expression and activity of the DNA damage and spindle-checkpoint proteins are frequently observed in cancer cells, and contribute to chromosomal instability (15). Thus, deregulated checkpoint pathways could cause an aberrant activation of CDK1. Indeed, over-expression of both cyclinB1 and CDC25, important regulators of CDK1 activity, are prognostic markers in colorectal and other cancers (18;21;42). In conclusion, CDK1SA-based analysis is a robust and useful assay to identify patients with a high risk of distant recurrence, who could benefit from adjuvant chemotherapy.

ACKNOWLEDGEMENTS

The authors wish to thank Alexandra Gnann, Sabine Leis, Gabi van Pelt and Inès Goossens-Beumer for their excellent technical assistance and for helpful discussion. The study was supported in part by Sysmex Corporation, Hyogo, Japan.

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CHAPTER 5

Combined markers of tumor cell proliferation and apoptosis are clinically prognostic in stage II colon cancer patients

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Submitted for publication in British Journal of Cancer

ABSTRACT

Background: TNM staging methods fall short identifying stage II colon cancer patients at high risk of distant metastasis who might benefit from adjuvant treatment. The addition of combined biomarkers representing important hallmarks of cancer, disruption of apoptosis and proliferation, to current staging criteria may provide a window for improvement.

Methods: The results of biochemical assays of enzymatic activity of caspase-3 and cyclin dependent kinase 1, representing the level of apoptosis and proliferation in tumor resection specimens of 217 stage II colon cancer patients, were combined into one parameter and correlated to tumor microsatellite status and patient outcome.

Results: Low levels of the proliferation marker tumor cell CKD1 activity and high levels of the apoptosis marker caspase-3 activity were independent predictors of better disease free survival (CKD1, hazard ratio 9.6, $p=0.029$; caspase-3 activity, HR 0.5, $p=0.03$). Within the tumor microsatellite stable (MSS) patient cohort, based on the combined results of these assays, 87.5% of the stage II patients that developed distant metastasis were correctly classified as high-risk.

Conclusion: Combined analysis of the level of proliferation and apoptosis was proven to be a reliable parameter to identify high-risk patients within stage II, MSS colon cancer patients. These results warrant clinical application after validation.

INTRODUCTION

Patient outcome in colon cancer highly depends on disease stage at time of diagnosis. This stage is determined using the TNM (Tumor Node Metastasis) criteria (1). Traditionally, the TNM stage is used to determine whether a patient is recommended to receive (neo-) adjuvant treatment. Nowadays, a disparity has risen between therapeutic decision-making and TNM staging. The statistics on colon cancer point out that within the stage II cohort a patient population can be identified with a high risk of distant metastasis, a risk comparable to patients diagnosed with stage III disease (2–4). Therefore, adjuvant chemotherapy is recommended for high risk patients with stage II colon cancer (5). Unfortunately, this ‘high-risk’ population cannot be identified using the TNM staging criteria alone. The clinical decision-making process therefore needs to be ‘updated’ with the addition of biomarkers to the traditional TNM criteria in order to make the identification of this high risk group possible.

Tumor development and progression partly depends on the disruption of the balance between cell proliferation and apoptosis, which under normal circumstances is responsible for tissue homeostasis (6,7). It has recently been shown that clinical outcome of colon cancer patients is related to the balance between cell proliferation and apoptosis at tumor level (manuscript submitted for publication) (8). Combined analysis of these markers might therefore be useful to detect the high-risk stage II patient population. In the current study we determined both the level of tumor proliferation as well as the level of apoptosis using a biochemical assay. The advantages of the use of biochemical assays are their highly accurate performance on only a minimal amount of fresh frozen tissue and furthermore their results are less influenced by inter-observer variety than for example immunohistochemistry (IHC) (9). More importantly, biochemical assays provide information on the functional activity of the enzyme *versus* solely expression levels of the protein, determined using IHC. To determine the level of tumor cell proliferation we used the Cyclin Dependent Kinase 1 Specific Activity (CDK1 SA) assay and to determine the level of apoptosis we used a caspase-3 activity assay. Both have been previously successfully tested for their applicability on colon cancer tissue. CDK1 SA for example has been shown to significantly predict distant metastasis in colon cancer patients and was significantly elevated in microsatellite-stable tumors. (10–12). The aim of this study was to combine the results of the biochemical analyses of apoptosis and proliferation in order to determine both the prognostic quality of this combined analysis as well as the discriminative power of these assays in the identification of high-risk stage II colon cancer patients.

PATIENTS AND METHODS

Patients

The study population consisted of 217 stage II colon cancer patients. All patients underwent surgical resection of their tumors between 1987 and 2006 at the Klinikum rechts der Isar, Technische Universität in München, Germany. None of the patients underwent any (neo-) adjuvant treatment. Of all patients, fresh frozen samples of their colon tumor specimens were collected. The tumor tissue was immediately dissected after surgery by an experienced pathologist and stored at -80°C . The median follow-up time was 7.9 years. The specimen collected were with informed consent, according to the vote of the Ethics committee of the Klinikum rechts der Isar (1927/2007).

Cyclin Dependent Kinase 1 specific activity assay

To determine the Cyclin Dependent Kinase 1 specific activity (CDK1 SA) an assay called C2P ('cell-cycle profiling'), which has been described previously, was used (12). In brief, from all patients 10-20 sections of $100\text{ }\mu\text{m}$ thickness were cut, analyzed for their tumor cell content and subjected to CDK1 SA analysis. CDK1 SA was calculated as cyclin dependent kinase (CDK) activity units ($\text{aU }\mu\text{l}^{-1}$ lysate) divided by its corresponding CDK expression units ($\text{eU }\mu\text{l}^{-1}$ lysate). Further details with respect to the CDK1 SA determinations in this specific cohort have been described previously (11).

Preparation of lysates and protein quantification

To determine the enzymatic activity of caspase-3 of each tumor sample 10 slides of $10\text{ }\mu\text{m}$ were cut and put in a 2.0 ml tube with $500\text{ }\mu\text{l}$ of lysis buffer (10 mM HEPES pH 7, 40 mM β -glycerophosphate, 50 mM NaCl, 2 mM MgCl_2 , 5 mM EGTA). Subsequently the samples were homogenized with a Polytron homogenisator (PT MR 2100, Kinematica AG, Lucerne, Switzerland) for 10 seconds and subjected to four freeze-thaw cycles that included storage in liquid nitrogen until samples were frozen followed by storage at 37°C until the samples were fully thawed. Until further processing, samples were stored at -80°C . Quantification of the total amount of protein in the lysates was performed using a Bradford assay (13). $10\text{ }\mu\text{l}$ of each sample was pipetted in a well of a 96 wells plate. Next, $300\text{ }\mu\text{l}$ of Coomassie Plus Reagent (Coomassie Blue Assay kit, Thermo Scientific, USA) was added to each well. The reagents were mixed in a plate shaker for 30 seconds and incubated for 10 minutes at room temperature. The absorbance levels were measured at 595 nm using a Benchmark Microplate Reader (Biorad, UK). Diluted bovine serum albumin (BSA, standard provided with Coomassie Blue Assay Kit, Thermo Scientific, USA) was used to create a calibration curve at protein concentrations ranging from $2000\text{ }\mu\text{g/ml}$ to $25\text{ }\mu\text{g/ml}$. A control sample containing no BSA was used to serve as a blank measurement. The absorbance level of this sample was subtracted from all measured absorbance levels to correct for background absorbance. All measurements were performed in duplicate.

Caspase-3 enzymatic activity determination

To determine the activity of the caspase-3 enzyme, a caspase-3-specific fluorometric assay was performed using Ac-DEVD-AFC (Sigma-Aldrich, USA) as substrate. For this assay an AFC calibration curve was prepared by diluting 1 mM of AFC (Sigma-Aldrich, USA) in reaction buffer (100 mM HEPES buffer pH 7.25, containing 10% (w/v) sucrose, 0.1% (v/v) Nonidet-P40 and 10 mM dithiothreitol (DTT, Sigma -Aldrich, USA) to reach a final AFC concentration ranging from 5000 nM to 2,5 nM. The emission at 505 nm levels of the calibration curve and tissue samples were analyzed in a 96-well microplate using a fluorometer (Victor³V 1420 Multi-label Counter, Perkin Elmer, USA) at excitation wavelength of 400 nm. Plotting the AFC emission levels against their concentrations in nM created the calibration curve. As a positive control of caspase-3 activation, a colorectal cancer cell line (SW480) was treated with 100 μ M Cisplatin. Lysates from the treated cells were prepared as described above. Each well to compose the calibration curve contained 100 μ l AFC standard sample, or for the detection of capase-3 activity 50 μ l tumor sample lysate and 50 μ l of the reaction buffer. 5 μ l (1 mM) of the Ac-DEVD-AFC (Sigma-Aldrich, USA) substrate solution was added to each well and the plate was incubated for 2 hours at 37 °C after which emission levels were measured. The emission level of a control sample containing 205 μ l of reaction buffer only was used for background correction. All measurements were performed in duplicate.

Caspase-3 enzymatic activity calculations

The AFC and the Bradford calibration curves both showed a correlation coefficient of 0.99. For each sample the protein concentration and the activity level of caspase-3 were determined by plotting the absorbance and fluorescence measurements in the calibration curves, making it possible to read out the corresponding protein concentration and caspase-3 activity. The enzymatic activity was then calculated as a ratio of the total amount of protein in each sample by dividing the determined AFC values by the protein concentration of the samples. This yielded an activity value expressed in pmol AFC/min/mg protein. The Bradford assay as well as the AFC value measurements were performed in duplicate. Samples were excluded from analysis when the difference between the two measurements was more than two times the standard deviation of the entire group of measurements. Additionally, samples that contained too little material for both measurements were excluded from analysis.

Microsatellite status determination

Five slides of 10 μ m of the fresh frozen tumor samples were cut for DNA isolation and subsequent determination of the microsatellite status. Of 191 patients tissue was available for this assay. DNA isolation was performed with the QIAampDNA™Mini Kit (QIAGEN Benelux, Venlo, The Netherlands) according to the manufacture's protocol. The DNA concentration and quality was checked with the ND-1000 NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The microsatellite status was determined with Promega MSI Analysis System, Version 1.2, which is

a fluorescence and multiplex PCR-based assay. In this assay 5 mononucleotide repeat markers, BAT-25, BAT-26, NR-21, NR-24, and MONO-27, were co-amplified to determine the microsatellite status. The results of this assay have been previously compared with the Bethesda panel markers and proven highly sensitive for microsatellite status determination (14). The results were checked and approved by an experienced pathologist.

Statistical analysis

The statistical analyses were conducted using the SPSS statistical analysis package version 17 UK. The One-Way Anova test and the Student's t-test were used to evaluate associations between the clinico-pathological variables, CDK1 SA and the caspase-3 activity levels. Survival analysis was performed using the standard Kaplan-Meier method to calculate survival probabilities and the log-rank test was applied to compare the survival curves. Disease Free Survival was defined as the time from date of surgery until disease recurrence (distant recurrence) or tumor-related death. Cox proportional hazards analysis was used for uni- and multivariate analysis. Test sensitivity was defined as the percentage of patients that developed a distant recurrence (DR) during follow-up correctly defined as high-risk. Test specificity was defined as the percentage of patients that did not develop a DR during follow-up correctly defined as low-risk. All statistical tests were two-tailed with a 0.05 significance level.

RESULTS

Patient characteristics, caspase-3 activity levels and CDK1 SA analysis

Tumor caspase-3 activity level could be successfully determined in 199 out of 217 patients. Eighteen patients (8.3%) were excluded from analysis because the measurements of the Bradford assay or the AFC value did not meet the quality control standards mentioned in the material and methods section. The characteristics of the successfully measured patients are summarized in table 1 and did not statistically significantly differ from the entire 217 patients included in this study. The mean caspase-3 activity level was 46.4 pmol AFC/min/mg protein with a range between 0 and 404.5 pmol AFC/min/mg protein. In concordance with previously published data, the dispersion of our results was skewed (15) Therefore, the median level of activity (24.4 pmol AFC/min/mg protein) was used as the cut-off value for group division into above-median caspase-3 activity (high) and below-median caspase-3 activity (low). The clinicopathological characteristics listed in table 1 were also analyzed for their association with the level of caspase-3 activity using this cut-off value. None of these showed a significant relation with the level of caspase-3 activity except for tumor microsatellite status. The tumor microsatellite stability status could be determined in 181 out of the 199 patients (91%), in the other cases to little tumor material was available for analysis. The percentage of patients with microsatellite stable (MSS) tumors was statistically significantly higher in the

Table 1

baseline characteristics of the stage II colon cancer patient population related to the caspase-3 activity assay

Patient characteristics	Number of patients (%) N=199	Caspase-3 activity mean	p-value
Gender			0.525
Male	133 (57)	48.7	
Female	86 (43)	43.5	
Age (median: 64 years)			0.279
Below median	100(50)	42.0	
Above median	99(50)	50.9	
Location primary			0.163
Colon Ascendens	53 (27)	55.7	
Colon Transversum	21 (10)	55	
Colon Descendens	24 (12)	32.7	
Coecum	30 (15)	58.4	
Sigmoid	71 (36)	36.6	
Tumor diameter (median: 5 cm)			0.122
Below median	101 (51)	40.2	
Above median	98 (49)	53	
T-stage			0.95
T3	173 (87)	46.5	
T4	26 (13)	45.8	
Differentiation grade			0.138
Well	1 (0,5)	9.7	
Moderate	134 (67)	41.3	
Poor	59 (30)	61.8	
Undifferentiated	2 (1)	30.4	
Missing	3 (1,5)		
Mucinous differentiation			0.573
Yes	21 (11)	44.3	
No	130 (65)	52.4	
Missing	48 (24)		
Tumor microsatellite status			0.000
MSS	131 (66)	33	
MSI	50 (25)	80	
Missing	18 (9)		
CDK1 SA (cut off: 11)			0.373
Below cut off	88 (44)	50	
Above cut off	111 (55)	43	

Abbreviations: Cm, centimeters; MSS, Micro Satellite Stable; MSI, Micro Satellite Instable; CDK1 SA, Cyclin-Dependent Kinase 1 Specific Activity.

This table shows the association of clinico-pathological characteristics with caspase-3 activity of the 199 primary stage II colon cancer patients. Caspase-3 activity was measured in pmol AFC/min/mg protein, CDK1 SA was measured in milli-activity unit per expression unit, maU eU⁻¹. The clinico-pathological characteristic did not differ significantly from the entire patient population of 217 patients.

low caspase-3 patient population compared to the high caspase-3 group (91.2% vs. 53.3%, P-value <0.001).

CDK1 SA measurements were available for all but 1 patient, in this case to little tumor material was available for analysis. The cut off level of high and low CDK1 SA was set at 11 (milli-activity unit per expression unit, maU eU⁻¹), based on a previously published study in which these activity level has been determined to be the optimal cut off level using bootstrap analysis (11). CDK1 SA was not significantly related to caspase-3 activity levels. In concordance with the previous published results CDK1 SA was not significantly related in direct comparison to the tumor microsatellite status (11).

The prognostic value of caspase-3 activity and CDK1 SA for disease free survival

The level of caspase-3 activity in tumor showed a significant relation to the patients' disease free survival (DFS). Patients with high levels of activity had a mean DFS time of 14.2 years compared to 13.3 years in the low activity level group (p-value: 0.012). The same was observed for the CDK1 SA. Patients with CDK1 SA below the cut off value had a mean DFS time of 14.8 years compared to a mean DFS time of 13 years in the group with CDK1 SA above the cut off value (p-value: 0.001, figure 1). When the survival analysis was stratified based on tumor microsatellite status this relation was only present in the MSS patient population. In this cohort, again high caspase-3 activity levels (p-value: 0.029) and low CDK1 SA levels (p-value: 0.005) were significantly related to a better DFS; this was not the case in the MSI population.

Table 2 shows the results of the Cox proportional univariate analysis for DFS. Both the caspase-3 activity level and the CDK1 SA were statistically significant prognostic indicators of DFS in univariate analyses. To determine the independent prognostic value of both caspase-3 activity level and CDK1 SA, multivariate analysis was performed taking into account known prognostic factors in colon cancer such as tumor microsatellite stability status. Both caspase-3 activity and CDK1 SA remained statistically significant, prognostic parameters in multivariate analysis, with a HR of 0.513 with a p-value of 0.03 and a HR of 9.6 with a p-value of 0.029, respectively. Additional multivariate analysis was performed to investigate the additional effect of microsatellite status through the implementation of an interaction term. This analysis showed no statistical significance. Therefore, the effects of CDK1 SA and caspase-3 activity on patient outcome are not influenced by the tumor microsatellite status.

The combined analysis of tumor proliferation and apoptosis level to identify high-risk stage II colon cancer patients

The goal of this study was to prove that the combined analysis of the level of tumor apoptosis and proliferation improves our ability to identify high-risk stage II colon cancer patients. To study this hypothesis we combined the results of both of our assays into one descriptive variable creating four groups. A double-positive

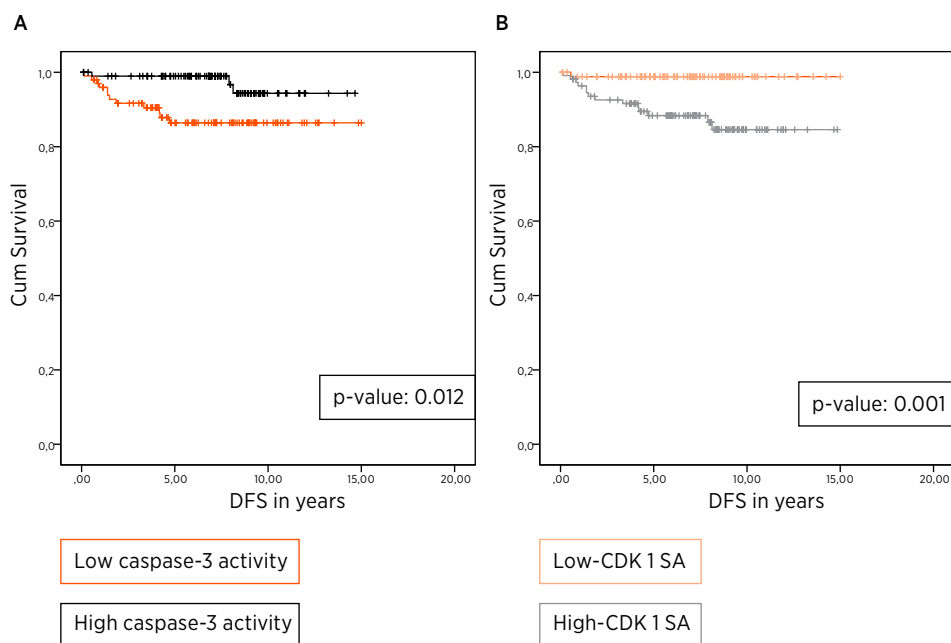


Figure 1

Abbreviations: DFS, disease-free survival; CDK1 SA, cyclin-dependent kinase 1 specific activity. Kaplan Meier curves for DFS in 199 stage II colon cancer patients according to level of (A) caspase-3 activity: high level of tumor caspase-3 activity is significantly related to a better DFS cancer patients; and (B) CDK1 SA: low level of tumor CDK1 SA is significantly related to a better DFS.

group, consisting of patients with tumors showing high level of apoptosis as well as proliferation; a double-negative patient group, showing low level of both apoptosis and proliferation; and two groups of patients of which one the tumor samples showed high level of proliferation and low level of apoptosis and one *vice versa*. We performed the same statistical analysis with this combination variable as previously described for both the assays separately. These results pointed out that patients who showed low levels of apoptosis but high levels of proliferation in their tumor samples had the worst outcome perspectives regarding DFS ($p < 0.001$) compared to all the other groups (figure 2). We performed the same analysis stratified for tumor microsatellite status (figure 2). From this analysis we concluded that our combination variable can be used to significantly determine outcome in the tumor MSS stage II patient cohort. We classified the low-caspase-3 activity and high-CDK1 SA population consisting of 53 (40%) patients of the entire MSS population as high-risk population, the remaining 78 (60%) as the low-risk population. In 2004 the American Society of Clinical Oncology (ASCO) listed a number of criteria that clinicians can use to identify the high-risk stage II colon cancer patients who might benefit from adjuvant chemotherapy (5). Although these criteria are only considered to be recommendations, some countries, like the Netherlands and Germany have adopted them in their national guidelines of the treatment of colon cancer patients. Our clinico-pathological dataset contained information on two of

Table 2

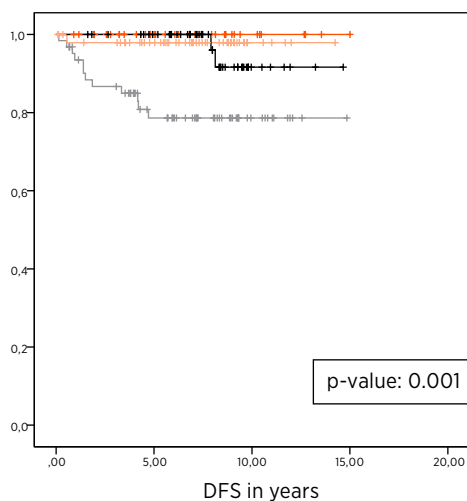
The Cox univariate analysis for disease-free survival.

Variables	Number of patients (%) N=199	Hazard Ratio	p-value
Gender			0.418
Male	133 (57)	1	
Female	86 (43)	0.641	
Age (median:64)			0.636
Below median	100(50)	1	
Above median	99(50)	0.779	
Location primary			0.862
Colon Ascendens	53 (27)	1	
Colon Transversum	21 (10)	0.644	
Colon Descendens	24 (12)	0.581	
Coecum	30 (15)	0.648	
Sigmoid	71 (36)	0.729	
Tumor diameter (median: 5cm)			0.849
Below median	101 (51)	1	
Above median	98 (49)	0.906	
T-stage			0.995
T3	173 (87)	1	
T4	26 (13)	1.005	
Differentiation grade			0.862
Well/Moderate	135 (68)	1	
Poor/Undifferentiated	61(30,5)	2.315	
Missing	3 (1,5)		
Mucinous differentiation			0.194
No	130(65)		
Yes	21(11)	1	
Missing	48 (24)	6.282	
Microsatellite status			0.166
MSS	131 (66)	1	
MSI	50 (25)	0.235	
Missing	18 (9)		
CDK1SA (cut off; 11)			0.021
Below cut off	88 (44)	1	
Above cut off	111 (56)	10.906	
Caspase-3 Activity (median 24,4)			
Below median	100(50)	1	
Above Median	99(50)	0.477	0.022

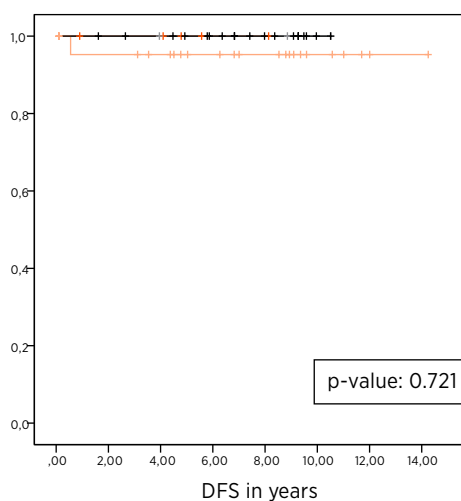
Abbreviation: N, total number of patients included in the analysis; HR, Hazard Ratio; P, p-value; MSS, Micro Satellite Stable; MSI, Micro Satellite Instable; CDK1SA, Cyclin Dependent Kinase 1 Specific Activity.

Cox univariate analysis for disease free survival. The significant p-values are marked bold. Caspase-3 activity was measured in pmol/AFC/min/mg protein, CDK1 SA was measured in milli-activity unit per expression unit, maU eU⁻¹.

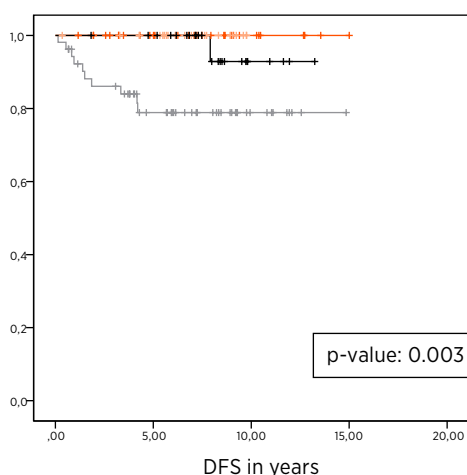
A Total population



B MSI cohort



C MSS cohort



Low caspase-3 and low-CDK1 SA

High caspase-3 and low-CDK1 SA

High caspase-3 and high-CDK1 SA

Low caspase-3 and high-CDK1 SA

Figure 2

Abbreviation; DFS, disease free survival; CDK1 SA, cyclin dependent kinase 1 specific activity; MSI, microsatellite instability; MSS, microsatellite stability.

Kaplan Meier curves for DFS in years for the combined caspase-3 & CDK1 SA parameter in (A) the total study population, and separately analyzed stratified for tumor microsatellite status in (B) for the MSI cohort and in (C) for the MSS cohort.

the criteria mentioned in the ASCO recommendations: T-stage and the number of lymph nodes resected. Based on these two variables we were able to classify 109 of the 131 MSS stage II colon cancer patients analyzed as low-risk (83%) and the remaining 22 as high-risk (17%). Of these 131 colon cancer patients, 16 patients actually developed a distant recurrence (DR) during follow-up. Based on our combined variable of caspase-3 activity and CDK1 SA levels we identified 14 out of these 16 patients correctly as high-risk, hence only two patients (12.5% of the high-risk patients) were incorrectly classified as low-risk. Using the ASCO recommendations, 11 out of these 16 patients were correctly classified, and 5 (31% of the high-risk patients) patients were incorrectly classified as low-risk. Based on these numbers the sensitivity of our combined variable was 88% with a specificity of 66%. Application of two the ASCO recommendations showed a lower sensitivity of 68%, but at a higher specificity of 85%.

DISCUSSION

This study underlines the clinical importance of analyzing both tumor apoptosis and proliferation for the identification of a patient cohort at high risk of distant metastasis within a stage II colon cancer population. We showed that a variable, based on the combined measurements of the level of tumor apoptosis measured with a biochemical caspase-3 activity assay and the level of tumor proliferation measured with a CDK1 SA assay, has substantial discriminating power to identify high risk stage II MSS colon cancer patients that might benefit from adjuvant therapy. Our variable performed equally to a subset of ASCO criteria designed to identify this patient selection at risk of distant metastasis in our MSS stage II colon cancer patient population (5).

Several countries, including the Netherlands, in their national guidelines, have adapted the ASCO criteria. Recent data from the Dutch Surgical Colorectal Audit (DSCA) pointed out that in the Netherlands only 18% of the stage II patients indicated as high-risk based on these recommendations actually received adjuvant treatment (16). From these data it can be concluded that the recommendations are not yet standard of care in the Netherlands, even though they are mentioned in the national guidelines. Furthermore, the criteria listed by the ASCO are all clinical and/or pathological parameters, no biomarkers were recommended for clinical use. It is our opinion that to better identify high-risk stage II colon patients the implementation of both clinical and pathological parameters as well as biomarkers should be applied. In other fields of medical care, such as in cardiovascular medicine, a positive effect on high-risk patient identification by combining biomarkers with established clinico-pathological parameters has already been proven (17). Until now both the ASCO's Tumor Markers Expert Panel and its European counterpart; The European Group on Tumor Markers, based on extensive reviews of the available literature, have not found any single biomarker to be of sufficient prognostic or predictive value for clinical application (18–20). We have recently proven that

clinical outcome of colon cancer patients greatly depends on the balance between cell proliferation and apoptosis at tumor level determined with IHC (manuscript submitted for publication). Both disruption of the pathway of apoptosis and the pathway of proliferation are important hallmarks of the highly regulated process of tumorigenesis (7,21). This current study validates the result of our previous study, only now using biochemical assays, proving the clinical significance of a combined parameter reflecting both tumor apoptosis and proliferation levels on patient outcome. Because we were able to validate the prognostic value of our parameter in the independent cohort in this current study and because our criteria showed to perform equally to a subset of the already clinically approved and applied ASCO criteria, future studies in independent cohorts will hopefully confirm that the use of our parameter together with the ASCO criteria will provide a synergistic effect in high-risk patient identification.

Interestingly, our parameter performed the best in high-risk patient identification in the MSS patient population. Microsatellite instability is predominantly found in right-sided tumors. These tumors display a phenotype that has been found to cause a substantial immune reaction resulting in higher levels of apoptosis and, moreover, tumor microsatellite instability has been significantly related to better patient outcome (22-24). Furthermore, a lack of benefit in DFS and OS has been shown by, amongst others, Ribic *et al.* with fluorouracil-based adjuvant chemotherapy in MSI patients (25,26). We feel that the results of these studies justify the application of our parameter in only a MSS stage II colon cancer patient cohort. Because they point out that the role of MSI status as a predictive marker for chemotherapy efficacy needs prospective validation and administration of chemotherapy in MSI Stage II CRC patients is controversially. Therefore it will be mainly within the MSS cohort that high risk patient that might benefit from adjuvant therapy.

In conclusion, current staging criteria have shown to be inadequate to determine a patient's need for adjuvant therapy especially those with a high risk of DR in stage II colon cancer. To improve these criteria, they should be re-evaluated which will implicate the addition of new biomarkers. This study indicates that the combination of the tumor apoptotic status, determined with a caspase-3 activity assay, and the determination of the tumor proliferation level, determined with a CDK1 SA assay, in especially MSS patients is a valuable tool in predicting distant recurrence in stage II colon cancer patients. Future validation of these assays for clinical application will enforce the reliability and clinical practicability.

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PART TWO

TUMOR IMMUNE INTERACTIONS

CHAPTER 6

Colorectal cancer vaccines in clinical trials

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Vaccines. 2011; 10 899-921

ABSTRACT

This review elucidates current strategies of active immunotherapy for colorectal cancer patients with a focus on T-cell mediated immunotherapy. Poor prognosis of especially stage 3 and 4 colorectal cancer patients emphasizes the need for advanced therapeutic intervention. Here we refer to clinical trials using either tumor cell-derived vaccines or tumor antigen vaccines with a special interest on safety, induced immune responses, clinical benefit and efforts to improve the clinical impact of these vaccines in the context of colorectal cancer treatment.

INTRODUCTION

Colorectal adenocarcinoma is the third most common cancer and accounts for a significant number of cancer deaths worldwide (1-3). The lifetime risk for colorectal cancer is about 5-6%, with a peak incidence in the 7th decade. Surgery is the treatment of choice when the disease is only confined to the bowel wall. However, 30-40% of patients have loco-regionally advanced or metastatic disease which cannot be cured by surgery alone. Adjuvant chemotherapy is indicated for stage III colon cancer (4-6). There is evidence to suggest that patients with a high risk group of stage II colon cancer may benefit from adjuvant chemotherapy as well (4-6). Patients with operable rectal cancer receive short-course preoperative radiotherapy (7,8). More than half of the patients that are initially believed to be cured by surgery and possibly adjuvant therapy develop recurrences and die of the disease (9). This prompts for more advanced treatment options and much effort has been put in exploiting the activation of the patient's immune system as a therapeutic modality by the enhancement of tumor-immunity.

IMMUNOTHERAPY OF CANCER

The tumor immune surveillance hypothesis postulated that the immune system identifies cancerous and/or precancerous cells and eliminates them before they can cause harm (10). In humans the majority of the evidence that supports the immune surveillance hypothesis comes from the observed increased relative risk ratios for various types of cancers with no apparent viral origin in immunosuppressed transplant recipients (11). Furthermore, patients on chronic immunosuppression with a colorectal tumor have significantly worse long-term oncologic outcomes after surgery (12). Moreover, the positive association between the number of tumor infiltrating lymphocytes and prognosis of colorectal cancer patients supports a role for the immune system in controlling colorectal cancer (13). These data support the development of strategies to use the immune system as anti-cancer therapeutic to prevent the growth of tumor recurrence and prolong survival of cancer patients. For a long time T-cell based immunotherapy of cancer patients was associated with limited clinical success. Recent studies have revealed the dawn of a new era in which the activation of tumor-specific T-cells starts to make a difference. Sipuleucel-T is the first therapeutic cancer vaccine to demonstrate effectiveness in Phase III clinical trials by prolonging the life of advanced or late stage metastatic, asymptomatic hormone refractory prostate cancer patients (HRPC) (14,15). The vaccine was approved by the U.S. Food and Drug Administration to treat patients with HRPC (16). Treatment with Ipilimumab, a monoclonal antibody that targets the immune regulatory molecule CTLA-4 represents the first modality that had a significant impact on the overall survival of patients with metastatic melanoma (17). These results are the first positive demonstration that blockade of a T-cell activity inhibitory pathway can be an effective cancer treatment.

Also adoptive T-cell therapy (ACT) has been found to be effective in the treatment for metastatic melanoma patients (18-20). In a recent series of three consecutive clinical trials in these patients, the infusion of autologous tumor infiltrating lymphocytes (TIL) yielded objective response rates between 49% and 72% (18-20). Last but not least, vaccination with a synthetic long-peptide (SLP) vaccine against the HPV-16 oncoproteins E6 and E7 resulted in the complete regression of human papillomavirus-16-positive, grade 3 vulvar intraepithelial neoplasias in 47% of the patients (21). Complete responses in this study were correlated with the strength of HPV-16-specific immunity (21). These encouraging results in patients with different types of carcinomas positively stimulate research on immunotherapy of colorectal cancer patients. This review provides an overview of clinical vaccination studies performed in patients treated for colorectal cancer and speculates on the development of therapeutic and prophylactic vaccination for colorectal cancer patients.

THERAPEUTIC VACCINATION FOR COLORECTAL CANCER

Both spontaneously- and therapy-induced cell-mediated tumor-specific immune responses need to be robust in order to attack and eliminate tumor cells. This calls for close collaboration between cells of the innate immune system and cells of the adaptive immune system (22). The cells of the adaptive immune system consist of B-cells and T-cells. Tumor-infiltrating B-cells sustain and enhance T-cell responses by producing antibodies (Abs), stimulatory cytokines, and chemokines, as well as serve as local antigen presenting cells (APCs) (23). Furthermore, they are involved in organizing the formation of tertiary lymphoid structures to sustain long-term immunity (23). T-cells recognize antigens that are presented in the context of MHC class I and II molecules at the surface of tumor cells. Immunotherapy should initiate responses against those antigens that uniquely target tumor cells. An important consideration is whether to use vaccines based on defined antigens or tumor cell derivatives.

INDUCTION OF ANTITUMOR IMMUNITY BY TUMOR CELL-DERIVED VACCINES

Tumor associated antigens (TAA) are poorly characterized and many remain to be identified. It is believed that the best source of antigens is the tumor cell itself. A possible benefit of tumor cell-derived vaccines is that, although targets for the adaptive immune system are unidentified, these preparations induce responses against multiple targets, possibly minimizing the chance for immune escape. A potential disadvantage of tumor cell-derived vaccines is that relevant TAA might be under-expressed in the tumor cell and thus results in weak immune responses compared to the injection with identified antigens. Furthermore, without the knowledge of the antigens presented to the immune system the possibilities of

immune monitoring with these vaccines are limited, thus prohibiting further clinical development based on immune parameters (24). Both autologous and allogeneic tumor cell-derived samples have been used in vaccination studies.

Reinjection of irradiated autologous tumor cells

Vaccine preparations based on autologous tumor tissue often consist of autologous single tumor cell suspensions. They are prepared from parts of the resected tumor and cryopreserved until use. Before reinjection, autologous tumor cell-derived vaccines are irradiated to prevent growth and combined with an immune stimulating agent such as BCG (25-29), Ulster strain of the Newcastle disease virus (NDV) (30-32), heat shock proteins (33) or IL-2 transfected fibroblasts (34) (table 1). These vaccines rely on boosting tumor-specific T-cells via tumor cell surface-expressed HLA-tumor peptide complexes. Furthermore, tumor antigens released by dying tumor cells may be presented to T cells by professional APCs. Professional APCs (e.g. DC) express very high levels of co-stimulatory molecules which is required to maximize T-cell activation (35). An advantage of using autologous tumor cell preparations is that all antigens that are presented to the immune system are also relevant for the recognition of the tumor. However, the preparation of such tailor-made vaccines is time consuming, relatively costly and, therefore, clinically difficult to apply outside a clinical trial setting. The use of these types of vaccines has revealed no serious toxic events (table 1). As TAA in this approach are unknown no extensive immune monitoring was performed. One study reported a T-cell response that was either a *de novo* induced or boosted in 15 of 29 patients (33). Other studies used the delayed cutaneous hypersensitivity skin reaction as an indirect parameter to measure vaccine specific immunity (table 1). In two studies a positive correlation was found with survival (28,31). Of the eight clinical phase II (27,31,32) and phase III (28,25,26,29,30,31) studies in different patient cohorts, six determined the effect on prognosis. Three studies reported that tumor cell reinjection combined with BCG had an effect on survival in subgroups (only stage II patients (25,26) or only patients with colon cancer (29)). Furthermore, one randomized phase III trial where NDV-infected autologous tumor cells were injected reported a significant effect on survival when compared to non-vaccinated patients (table 1) (31). Other phase II and randomized phase III trials using NDV-infected autologous tumor cells, described a positive effect on survival of vaccinated patients, when compared to historical controls or in subgroups of only colon cancer patients (table 1) (31,32). Despite these results with autologous tumor cell-derived vaccines no new clinical trials have been initiated. This is possibly due to the lack of pharmaceutical support for tailor-made vaccines and the problems to optimize the vaccines based on limited immunological data.

Table 1

Vaccine trials using either autologous or allogeneic tumor cell-derived samples

Year(s) of publica- tion + Reference Citation(s)	Type of study	Type of cancer	No. of patients: enrolled; treated; control	Toxicity	Immune response	Impact on survival	Concurrent therapy ^A
<i>Studies of irradiated autologous tumor cell vaccines & BCG</i>							
Uyl-de Groot <i>et al.</i> and Vermorken <i>et al.</i> 2005 (25); (1999) (26)A	Randomized phase III trial (ASI/BCG or no therapy after resection)	Stages II/III colon cancer	254; 128; 126	No patient refused vaccination because of side-effects, and none needed to be admitted	Increasing indurations during vaccination; 92% of the patients > 10 mm induration to the fourth vaccine	Overall no impact on prognosis; subgroup analysis stage II vaccinated patients a significant beneficial effect on: OS, RFS, RFI	None
Baars <i>et al.</i> 2002 (27)	Phase II trial	Stage III colon cancer	104; 53; 0	Grade III/IV reaction comparable to chemotherapy alone	In each patient local erythema and induration after the third and fourth vaccinations, despite chemotherapy in between third and fourth vaccinations	Not described	Chemotherapy (5-FU + Leucovorin)
Harris <i>et al.</i> 2000 (28)	Randomized phase III trial (ASI/BCG or no therapy after resection)	Stage II/III colon cancer	412; 182; 188	79% of vaccinated patients local reaction at vaccination site	106 of the 150 vaccinated patients had a positive DCH response to the third vaccine	No survival difference between treatment arms; In vaccination arm the magnitude of the DCH response correlated with improved prognosis	None
Hoover Jr <i>et al.</i> 1993 (29)	Randomized phase III trial (ASI/BCG or no therapy after resection)	Dukes' stage B2-C3 colorectal cancer	89; 41; 39	No serious side effects	16 of 20 tested immunized patients became positive for DCH after vaccination	Overall no impact on survival (OS and DFS); subgroup analysis advantage OS and DFS vaccinated colon cancer patients	rectal cancer patients received postimmunotherapy radiation

Table 1

Continued

Year(s) of publication + Reference Citation(s)	Type of study	Type of cancer	No. of patients: enrolled; treated; control	Toxicity	Immune response	Impact on survival	Concurrent therapy ^A
<i>Studies of NDV-infected autologous tumor cell vaccines</i>							
Schulze <i>et al.</i> 2008 (30)	Randomized phase III trial (ASI/NDV or no therapy after resection)	Complete resected liver metastases from colorectal cancer	51; 25; 26	No serious side effects	Not described	Overall no impact on prognosis; subgroup analysis advantage OS and DFS vaccinated colon cancer patients compared to control group	None
Liang <i>et al.</i> 2003 (31)	Phase II trial	Stage IV gastro-intestinal tumors	25; 25; 0	Not described	Not described	1 complete response, 5 partial responses	None
	Randomized phase III trial (ASI/NDV or no therapy after resection)	Stage I-IV colorectal cancer	567; 310; 257	Not described	90 % of patients resected and vaccinated showed a positive DTH reactions, whereas in resection alone group all negative	Higher mean and median survival for vaccination group compared to the resection group alone	None
Ockert <i>et al.</i> 1996 (32)	Phase II trial	Stage II/III colorectal cancer	57; 48 ^B ; Historical controls	No serious side effects	Of 31 patients tested, 21 showed an increasing amount of DTH reactivity, 8 showed a positive DTH response during vaccination	Improved overall survival in compared to patients from a historical control group	None

Table 1

Continued

Year(s) of publication + Reference Citation(s)	Type of study	Type of cancer	No. of patients: enrolled; treated; control	Toxicity	Immune response	Impact on survival	Concurrent therapy ^A
<i>Other studies using autologous tumor cell-derived vaccines</i>							
Mazzaferro <i>et al.</i> 2003 (33)	Phase I trial using autologous HSPPC-96 tumor cell-derived vaccine	Complete resected liver metastases from colorectal cancer	29; 22; 0	No relevant toxicity was observed	Either a <i>de novo</i> induced or a significant increase of preexisting class I HLA-restricted T-cell-mediated anti-colon cancer response was observed in 15 of 29 patients; frequency of CD3 ⁺ , CD45RA ⁺ , and CCR7 ⁺ T lymphocytes increased in immune responders	Occurrence of immune response led to better tumor-free survival, independent of predicted prognosis	None
Sobol <i>et al.</i> 1999 (34)	Phase I trial using autologous tumor cells and autologous fibroblasts genetically modified to IL-2 (dose escalating)	Metastases from colorectal cancer	10; 8; 0	Fatigue and/or flu-like symptoms were experienced by 7 of 10 patients	5 of 10 patients DTH response at the sites of the second or subsequent vaccinations; there was a 5-fold increase following treatment in the frequency of tumor cytotoxic T-cell precursors in 2 of 6 evaluable patients	All patients eventually developed progressive disease	None

Table 1

Continued

Year(s) of publication + Reference Citation(s)	Type of study	Type of cancer	No. of patients: enrolled; treated; control	Toxicity	Immune response	Impact on survival	Concurrent therapy ^A
<i>Allogeneic (MAGE positive) cell lysate-pulsed autologous dendritic cell vaccine</i>							
Toh <i>et al.</i> 2009 (37)	Phase II trial	MAGE positive stage IV colorectal cancer	26; 20; 0	Only grade I/II toxicity	9 of 12 patients positive DCH reaction; DC vaccination down regulates peripheral Treg	One patient partial response; seven stable disease, clinical benefit response rate of 40%	None
Burgdorf <i>et al.</i> 2009 (138); and Burgdorf <i>et al.</i> 2008 (36)	Phase II trial	Stage IV colorectal cancer	20; 17; 0	No serious adverse events	No positive DCH responses;	Stable disease in 24% (4/17) of the patients; stable disease correlated with increasing levels of plasma GM-CSF, TNF- α , IFN- γ , IL-2, and IL-5	None

Abbreviations: 5-FU: 5-Fluorouracil; ASI: active specific immunotherapy; BCG: bacillus Calmette - Guérin; DCH: delayed cutaneous hypersensitivity skin testing; DFS: disease free survival; DTH: delayed-type hypersensitivity; GM-CSF: Granulocyte-Macrophage Colony Stimulating Factor; HSPPC-96: Heat shock proteins peptide-complex gp96 isolated from autologous tumor cells; IFN- γ : Interferon- γ ; IL: interleukin; No.: number; OS: overall survival; RFS/RFI: Recurrence Free Survival/Interval; TNF- α : Tumor necrosis factor- α ; Treg: regulatory T-cells.

^AChemotherapy, radiation therapy, hormonal therapy, or cytokine therapy given/allowed at the same time as vaccine therapy; ^B only 48 patients were treated with NDV-infected tumor cells, the remaining patients were treated with BCG to compare effects.

Dendritic cells loaded with an allogeneic tumor cell vaccine

Another approach is to use directly *ex vivo* antigen-pulsed DCs for vaccination. Support for this strategy comes from animal studies. Here it was demonstrated that such DC administered to tumor bearing hosts were able to elicit effective antitumor T-cell immunity. DCs can be easily generated from the blood of patients and, therefore, this approach was translated to clinical trials designed to study their capacity to prime tumor-specific CD8⁺ T-cells and T-helper cells and clinical efficacy. The advantage of allogeneic tumor cells to pulse DCs is that the allogeneic tumor cell component can become an off-the-shelf product. A potential disadvantage is that the immune system will be aroused against tumor antigens that are not present in the patient's tumor, and as such are irrelevant. In a number of trials DCs were pulsed with an allogeneic melanoma cell lysate, which amongst others is rich in the cancer/testis antigen MAGE (36,37), the expression of which has not been found in normal tissues except for the testis. Approximately 40% of all colorectal carcinomas expressed at least one of MAGE A-1 to -6 antigens (38-41). No severe adverse events were found in these clinical trials but the clinical effect has been limited to the description of cases (table 1) (36,37). In addition, the induction of anti-tumor immunity was not studied in great detail and mainly relied on the description of delayed cutaneous hypersensitivity skin tests. A large study focusing on survival is lacking.

TUMOR ANTIGENS STUDIED IN CLINICAL TRIALS

Defined antigens to be used as vaccine candidates ideally should be overexpressed in the context of HLA at the cell surface of tumor cells and not (or at very low) levels by other cells of the human body. All antigens used in vaccination studies for colorectal cancer comprise TAA and consequently are likely to be expressed by normal cells (42-44). Different TAA such as: p53, CEA, MUC1, Sialyl-Tn, 5T4, SART3, MAGE have been applied in clinical trials to vaccinate colorectal cancer patients (table 2) (42-46). The use of antigens potentially expressed by normal cells bears the risk of immune tolerance. Indeed, tolerance to many TAA has been found. For instance, animal models indicated that the p53-specific CD8⁺ T-cell but not the CD4⁺ T-cell repertoire is severely restricted by self-tolerance and might only consist of lower affinity p53-specific CD8⁺ T-cells (47,48). Data from our recent clinical vaccination trial with the p53-SLP vaccine confirmed these results (49). In contrast a blunted CD4⁺ T-cell repertoire was found for the T-cell response against the carcinoembryonic antigen (CEA) in animal models (50). Also studies in a MUC1-transgenic mouse model indicated that low antibody and CTL responses to MUC1 peptides are due to CD4⁺ T-cell tolerance (51-54). These results indicate that tolerance forms a potential hurdle for immunotherapies of cancer when using TAA. A possible option to bypass tolerance and to induce both CD4⁺ and CD8⁺ tumor specific T-cell responses consists of designing vaccines that induce responses

Table 2

Different types of tumor-associated antigens with examples of antigens used to vaccinate colorectal cancer patients

Type	Description	Tumor associated antigen
Differentiation antigens	expressed by the tumor and the normal tissue from which it derives	CEA 5T4 Ep-CAM
Shared or cancer testis antigens	expressed by tumor cells and not by normal tissues, with the exception of spermatogonia, which do not express HLA class I molecules and thus are not targeted by CTL	MAGE SSX-2 COA-1
Over expressed antigens	antigens not or in low amounts expressed in normal tissues, but massively over expressed by malignant cells	p53 EphA2 MUC2 Sialyl-Tn Sart3 Survivin WT1
Unique antigens	Mutation or genetic instability uniquely expressed by tumor cells	Frameshift mutated products RAS

Abbreviation: CTL: Cytotoxic T lymphocyte. Data from (42;139).

against different TAA able to induce complementary reactivity of non-tolerized CD4⁺ and CD8⁺ T-cells.

Different strategies have been used to immunize patients against the fore-mentioned TAAs. Four vaccination strategies have been popular and tested in mainly phase I and II trials i.e.: peptide vaccination, dendritic cell vaccination, polynucleotide vaccines and recombinant viruses.

Peptides

An elegant approach is the use of synthetic peptides that represent parts of the TAA and are in general injected intradermally or subcutaneously. Two types of peptide strategies are currently used for vaccination purposes: minimal peptide vaccines (55) and synthetic long peptides vaccines (56). Minimal peptide vaccines comprise the minimal cytotoxic T-cell peptide-epitope sequence that can bind directly to its presenting HLA class I molecule. Notably, each peptide can only bind to one specified HLA class I subtype and as such vaccination is limited to those patients expressing that specific type of HLA molecule. Direct injection of such peptide vaccines into patients have met with limited clinical success, probably because the induced CD8⁺ T-cell responses do not possess strong effector functions, the response is transient because of the lack of T-cell help. In some vaccination strategies, these minimal peptide vaccines may induce immunological tolerance rather than immunity (55). Table 3 summarizes a large diversity of phase I and II studies using peptide vaccines. Minimal peptide vaccines used in

clinical trials were derived from different antigens: CEA (57), survivin (58), SART3 (59), and a personalized mixture of antigens (60-62) (table 3). These minimal peptide vaccines were all well tolerated with mainly grade I/II adverse events at the injection site (table 3). In most studies the peptide vaccines induced or boosted antigen-specific T-cell or IgG responses in the majority of vaccinated patients (table 3). Unfortunately, detailed insights into the phenotype and function of these T-cells was missing. Although phase I and II studies are not designed to describe therapeutical effects, reduction of tumor load was described in nearly all studies. One study described a positive relation between the presence of vaccine-induced IgG and overall survival (60), whereas others mentioned reduction of tumor mass or a decrease in the level of serum tumor markers in individual patients (table 3). To gain the induction of strong T-cell responses the concept of overlapping synthetic long peptides (SLP) was introduced. These long peptides, containing a mix of T-helper epitopes and cytotoxic T-cell epitopes do not bind directly to MHC class I at the cell surface but are taken up by professional APCs such as DCs which process and present the appropriate epitopes in MHC class I and II molecules at the cell surface in the right context thereby activating both CD8⁺ and CD4⁺ T-cells and in the end a more effective immune response (56). Notably, the use of pools of overlapping peptides corresponding to the complete amino acid sequence(s) of one or more different antigens allows for the application of vaccines in groups of patients irrespective of their HLA type. The SLP vaccines have successfully been used to induce strong and broad CD4⁺ and CD8⁺ T-cell immunity in animals and humans (56,63). Long peptides vaccines used in phase I or II vaccination trials to vaccinate colorectal cancer patients represented the TAA: p53 (49), β HCG (64), MUC-1 (65) and mutation specific Ras (66) (table 3). Only limited toxicity has been found in these trials. Two of these four studies determined vaccine-induced immunity. One study, using a mutation specific Ras peptide, was able to establish mutated Ras-specific CD4⁺ T-cell and CD8⁺ T-cell responses (66). Our study on p53-SLP vaccination revealed long lasting vaccine-specific responses in 9 of 10 colorectal cancer patients as determined by IFN- γ enzyme linked immunospot, proliferation and cytokine bead arrays (49). Furthermore, p53-specific T-cells isolated from the vaccination site were characterized as CD4⁺ T-cells producing both T-helper type 1 and 2 cytokines on stimulation with p53 peptide and p53 protein (49). However, multiparameter flow-cytometry revealed that only a minor population of the p53-specific CD4⁺ T-cells was optimally polarized, suggesting that the vaccine strategy used was not optimal for the induction of p53-specific Th1 cells. These results emphasize the importance of immune monitoring to further optimize vaccine strategies and to better understand clinical responses after vaccination (49). Only one study, using a β HCG long peptide vaccine found that patients with high anti- β HCG antibodies levels exhibited a significant longer survival compared to patients who developed low anti- β HCG antibody levels (64). Unfortunately, no efficacy study was performed to determine the true effect of vaccination on survival. The immunogenicity of peptide vaccines may be further increased by using adjuvants such as: incomplete Freund adjuvant, cytokines, and/or agonists of innate immune receptors.

Table 3

Vaccine trials using peptide vaccines

Year(s) of publication + Reference Citation(s)	Type of Study	Vaccine	Type of Cancer	No. of Patients: Enrolled; Treated; Control	Toxicity	Immune response	Strongest Benefit Reported ^c	Concurrent Therapy ^a
<i>HLA class I minimal/peptide vaccines</i>								
Hattori <i>et al.</i> 2009 (60)	Phase I trial	Personalized vaccine (maximum of 4 peptides) HLA-A24-restricted or HLA-A2-restricted peptides: SART1, SART2, SART3, Lck, MRP3, PAP, PSA, Her2/neu, CEA, PTHrp, UBE2V, EIF4EBP, WHSC2, CypB, EGFR, PSCA admixed with Montanide ISA-51	HLA-A24 + or HLA-A2 + metastatic colorectal cancer	14	This therapy was well-tolerated (one patient grade-3 skin reaction at the vaccination site)	An increase in peptide specific IFN-γ production or peptide-specific IgG after the tenth vaccination was observed in nine of ten or eight of ten patients tested, respectively	IgG responses correlated with overall survival	(UFT or UZEL)
Sato <i>et al.</i> 2007 (61)	Phase I trial	Personalized vaccine (maximum of 4 peptides) HLA-A24-restricted or HLA-A2-restricted peptides: SART3, CypB, Lck, MRP3, PAP, EZH2, PSCA, PSA, WHS, HNR, MAP, UBE, EIF, EGFR, PSA, HER2/neu, CEA admixed with Montanide ISA-51	HLA-A24 + or HLA-A2 + advanced gastric or colorectal cancer	11 (7 CRC); 11; 0	Therapy was generally well tolerated, two patients grade 3 toxicity (anemia and neutropenia)	An increase in peptide-specific IgG was observed in 8 of 11 patients increase in peptide-specific IFN-γ production by CTL was observed in 7 of 11 patients, possibly depending on the dose of chemotherapy	4 patients stable disease, other 7 patients progressive disease	Chemotherapy (TS-1)

Table 3

Continued

Year(s) of publication + Reference Citation(s)	Type of Study	Vaccin	Type of Cancer	No. of Patients: Enrolled; Treated; Control	Toxicity	Immune response	Strongest Benefit Reported ^d	Concurrent Therapy ^a
Weihrauch <i>et al.</i> 2005 (57)	Phase I/II trial	HLA-A2 restricted CEA derived peptide (CAP-1) admixed with different adjuvants (GMCSF/IL-2, dSLIM/IL-2, or IL-2)	HLA-A2 + metastatic colorectal cancer	17; 12; 0	Six grade 1 local skin reactions and one mild systemic reaction to vaccination	Eight patients showed elevation of CAP-1 specific CTLs. Neither of the adjuvants provided superiority	five complete response, one partial response, five stable disease, and six progressive disease.	Chemotherapy ((irinotecan/ 5-fluorouracil / leucovorin)
Sato <i>et al.</i> 2004 (62)	Phase I trial	Personalized vaccine (maximum of 4 peptides) HLA-A24- restricted peptides: SART1, SART2, SART3, Lck, ART1, ART4, CypB admixed with Montanide ISA-51	HLA-A24 + advanced colorectal carcinomas	10; 10; 0	No severe adverse effect was observed	Increased CTL response to cancer cells was detected in post-vaccination PBMCs of five patients. Anti-peptide IgG became detectable in postvaccination sera of seven patients. Three patients developed a positive DTH response to at least one of the peptides administered	One patient was found to have a partial response; another had a stable disease, sustained through 6 months.	None
Tsuruma <i>et al.</i> 2004 (58)	Phase I trial	HLA-A24-restricted peptide, survivin-2B (AA 80-88)	HLA-A*2402 +, surviving +, advanced or recurrent colorectal cancer	17; 15; 0	Of 15 patients, three suffered grade I/II toxicities, no severe adverse events	increase in peptide-specific CD8+ T-cells after 4 vaccinations, in one patient tested	In 6 patients, tumor marker levels (CEA and CA19-9) decreased transiently during the period of vaccination; Slight reduction of the tumor volume was observed in one patient, other patients stable (3) or progressive (11) disease	None

Table 3

Continued

Year(s) of publication + Reference Citation(s)	Type of Study	Vaccin	Type of Cancer	No. of Patients: Enrolled; Treated; Control	Toxicity	Immune response	Strongest Benefit Reported ^c	Concurrent Therapy ^a
Miyagi <i>et al.</i> 2001 (59)	Phase I trial	HLA-A24 restricted peptides SART3 (AA 109-118; 315-323)	HLA-A24+ advanced colorectal cancer	12; 11; 0	No severe adverse events were associated with the vaccinations.	Significant levels of increased cellular immune responses postvaccination to both HLA-A24+ colon cancer cells (7 of 11) and the vaccinated peptide (7 of 10) were observed	Not described	None
<i>Long peptide vaccines</i>								
Speetjens <i>et al.</i> 2009 (49)	Phase I trial	10 overlapping long peptides representing the p53 protein (AA 70-248) admixed with Montanide ISA-51	metastatic colorectal cancer	10, 10, 0	Toxicity was limited to grade 1/2, mostly at the vaccination site.	Overall in 9 of 10 colorectal cancer patients T-cell responses were induced using comprehensive analysis with complementary assays: IFN- γ ELISPOT, proliferation assay, cytokine secretion, and multiparameter flow cytometry	Not described	None
Moulton <i>et al.</i> 2002 (64)	Randomized phase II trial (high to DT vs low dose)	CTP37-DT, 37-AA peptide from the COOH terminal end of β hCG conjugated	metastatic colorectal cancer	77; 77; 0	69% of the patients reported an mild adverse event	56 of the 77 patients developed anti- β hCG antibody, no difference between two dose groups	High anti- β hCG Ab levels exhibited a significant longer survival compared patients who developed low anti- β hCG antibody levels.	None

Table 3

Continued

Year(s) of publication + Reference Citation(s)	Type of Study	Vaccin	Type of Cancer	No. of Patients: Enrolled; Treated; Control	Toxicity	Immune response	Strongest Benefit Reported ^c	Concurrent Therapy ^a
Khleif <i>et al.</i> 1999 (66)	Phase I trial (dose escalation)	Mutation specific Ras peptide (AA 5-17) Ras-12Asp, Ras-12Cys or Ras-12Val	Advanced metastatic solid tumors	15 (10 CRC); 11 (8 CRC); 0	No serious acute or delayed side effects	2 of 10 vaccinated patients showed CD4 ⁺ T-cell mutation specific proliferative responses, 2 of 3 patients showed CD8 ⁺ T-cell responses	1 of 11 patients showed stable disease, other progressive disease	None
Goydos <i>et al.</i> 1996 (65)	Phase I trial	105 AA polypeptide MUC-1 peptide admixed with BCG	adenocarcinoma	63 (30 CRC); 63; 0	Most experiencing local ulceration at the vaccination site.	Only 3 patients had a strong skin response to the long peptide; 37 of 55 biopsies showed intense T-cell infiltration; 7 of 22 patients tested had a 2- to 4-fold increase in mucin-specific CTL.	Not described	None

Abbreviations: AA: amino acid; β -hCG: β -subunit of Human chorionic gonadotropin; CEA: carcinoembryonic antigen; CRC: colorectal cancer; dSLIM: CpG-containing DNA molecules; DT: diphtheria toxoid; DTH: delayed type of hypersensitivity; GM-CSF: granulocyte macrophage colony-stimulating factor; IFN- γ : Interferon- γ ; PBMC: Peripheral blood mononuclear cell; TS-1: 5-fluorouracil derivative; UFT: Uracil/tegafur;UZEL: Calcium folinate.

^aChemotherapy, radiation therapy, hormonal therapy, or cytokine therapy given/allowed at the same time as vaccine therapy.

Dendritic cell vaccines

The injection of autologous DCs pulsed with MHC class I restricted peptides derived from CEA (67-70), Her2/NEU (67), MAGE2 (67), MAGE3 (67,71), or DCs expressing antigen via recombinant techniques such as viral vectors (72) or mRNA encoding the CEA antigen (73) may form an alternative approach (table 4). In contrast to the use of defined HLA class I-restricted peptide-epitopes the latter approach has the advantage to include patients irrespective of their HLA type. A potential disadvantage is the possibility that the viral vector contains more immunogenic epitopes that compete for binding and presentation with the epitopes derived from the antigen of interest, thereby weakening the induction of tumor specific immunity. A series of phase I/II trials in colorectal cancer patients showed that DCs vaccines are safe and able to arouse a T-cell response (table 4). Comprehensive immune monitoring revealed that dendritic cell vaccines induce peptide-specific IFN γ -producing T-cells with variable efficiency. It is difficult to compare the outcomes of peptide vaccines with peptide-pulsed DCs vaccines as mostly the assays have not been harmonized and no gold standard exists. One study found that objective clinical responses correlated with the expansion of tetramer+CD8+ T-cells (70). The other studies described only cases (67,69,72,73,71,70). An interesting study reported the application of a multiple target vaccine (67) in order to prevent immune escape. Unfortunately, only immune reactivity against CEA was tested in this study limiting the interpretation of this trial (67).

Viral vector vaccines

Since the discovery that viral infection results in presentation of viral peptides in the context of MHC class I and II on infected cells, viruses have been optimized as vectors for the delivery of TAA. Vectors often used to express recombinant proteins in colorectal patients are: canarypox virus (ALVAC) and Modified Vaccinia Ankara (MVA) virus. Sometimes constructs to co-express co-stimulatory molecules such as B7.1 were used (74-76). In general, these vaccines are not toxic (table 5) and have been used to induce immunity against CEA (74-79), EpCAM/KSA (80), p53 (81,82), and 5T4 (83,84,85,86) (Table 5). The use of these vaccines not only yielded antibody and T-cell responses against tumor antigens but frequently also against viral components. In most cases the immune response to the tumor antigens was a bit disappointing. The foreign viral components may have formed a more attractive target for the immune system. Indeed, whereas we detected only low p53-specific T-cell reactivity in patients vaccinated with ALVAC-p53, a strong T-cell response against the viral vector was found (82). In contrast, the response to p53 was much stronger and broader when patients were vaccinated with p53 overlapping long peptides (49). Interestingly, the MVA-5T4 vaccine did induce strong 5T4-specific immune responses. The group of patients with stronger 5T4-specific proliferative responses showed significantly longer survival (85,86). Unfortunately so far only phase I and II studies have been performed. Therefore mainly descriptive effects on survival and tumor mass have been reported.

Table 4

Vaccine trials using Dendritic Cells

Year(s) of publication + Reference Citation(s)	Type of Study	Vaccin	Type of Cancer	No. of Patients: Enrolled; Treated; Control	Toxicity	Immune response	Strongest Benefit Reported ^c	Concurrent Therapy ^d
<i>Dendritic cell vaccine</i>								
Kavanagh <i>et al.</i> 2007 (67)	Phase I/II trial	Autologous DCs pulsed with peptides CEA605, D6; CEA691H5; HER-2/neu.369.V2V9; HER-2/neu.689; MAGE2.157; MAGE3.112.15 for HLA A0201	HLA-A2+ advanced colorectal cancer patients	21; 11; 0	No significant treatment-related toxicity was reported	Only CEA specific T-cell responses were tested to a CEA derived peptide and detected by ELISPOT in 3 of 11 patients	All patients showed progressive disease	None
Lesterhuis <i>et al.</i> 2006 (68)	Phase I trial	Autologous DCs pulsed with wt-CEA-peptide CAP-1 (CEA571-579) for HLA-A0201	HLA-A0201+ Liver metastases from colorectal cancer	10; 10; 0	No grade III/IV toxicities	In 7 of 10 high numbers of de-novo CEA-specific T-cells in post-treatment DTH biopsies which produced high amounts of IFN- γ ; in unstimulated peripheral blood no CEA-specific T cells were detected	Not described	Surgical resection
Babatz <i>et al.</i> 2006 (69)	phase I/II clinical trial	Autologous DCs pulsed with CEA peptide 610D (CEA605-613, 610D) for HLA A0201	HLA-A0201+ CEA+ metastatic cancer	9 (7 CRC); 9 (6 CRC); 0	Immunization was well tolerated by all patients without severe signs of toxicity.	ELISPOT IFN- γ producing CEAalt peptide-specific CD8 ⁺ T-cells in 5 of 9 patients; 3 of 9 CD8 ⁺ T lymphocytes recognizing the native CEA peptide	1 of 9 patients had stable disease for 4 months	None

Table 4

Continued

Year(s) of publication + Reference Citation(s)	Type of Study	Vaccin	Type of Cancer	No. of Patients: Enrolled; Treated; Control	Toxicity	Immune response	Strongest Benefit Reported ^c	Concurrent Therapy ^d
Morse <i>et al.</i> 2005 (72)	Phase I trial	Autologous DCs modified with fowlpox encoding CEA and co-stimulatory molecules (TRICOM)	CEA+ metastatic cancer	14 (11 CRC); 12; 0	No grade III/IV toxicities	Increase in the frequency of CEA-specific T-cells in PBMC of 10 patients using ELISpot	One patient decrease in the CEA level from 46 to 6.8 and a minor regression in adenopathy; five other patients were stable through at least one cycle of immunization	None
Morse <i>et al.</i> 2003 (73)	Phase I trial (dose-escalating)	autologous DCs loaded with CEA mRNA	CEA+ metastatic cancer	29 (11); 24; 0	immunizations were well tolerated	Not described	1 complete response, 2 minor responses, 3 with stable disease, and 18 with progressive disease of 24 patients.	None
Morse <i>et al.</i> 2003 (73)	Phase II trial	autologous DCs loaded with CEA mRNA	resected hepatic metastases of CEA+ colon cancer	13 (11); 13; 0	immunizations were well tolerated	Erythema at injection site in 6 of the 13 patients,.	9 of 13 patients have relapsed at a median of 122 days	None

Table 4
Continued

Year(s) of publication + Reference Citation(s)	Type of Study	Vaccin	Type of Cancer	No. of Patients: Enrolled; Treated; Control	Toxicity	Immune response	Strongest Benefit Reported ^c	Concurrent Therapy ^d
Sadanaga <i>et al.</i> 2001 (71)	Phase I trial	Autologous DCs pulsed with Mage-3 peptide for HLA-A2 or -A24	HLA-A2 or -A24+ Mage-3 expressing advanced gastrointestinal carcinomas	12 (3 colon); 9 (3); 0	no toxic side effects were observed	CTL responses after vaccination in four of eight patients	Tumor markers decreased in seven patients. In addition, minor tumor regressions evidenced by imaging studies were seen in three patients.	None
Fong <i>et al.</i> 2001 (70)	Phase I trial	Autologous DCs pulsed with CEA peptide 610D (CEA605-613, 610D) for HLA A0201	HLA A0201+, CEA+ metastatic or recurrent colon or nonsmall cell lung cancer	12 (10 CRC); 12; 0	No grade III/IV toxicities	Lytic activity was seen in 7 of the 12 patients; 5 of 12 patients had 610D-tetramer ⁺ CD8 ⁺ T-cells after vaccination	Objective responses were observed in 2 of the 12 patients and 2 of the 12 patients had disease stabilization; Clinical response correlated with the expansion of tetramer ⁺ CD8 ⁺ T-cells	Different doses Flt3 ligand, to expand DCs <i>in vivo</i>

Abbreviations: CEA: carcinoembryonic antigen; CRC: colorectal; DC: Dendritic Cell; PBMC: peripheral blood mononuclear cells; CEAlt: Altered HLA-A*0201-restricted nonamer CEA610D; CTL: Cytotoxic T lymphocyte; ELISpot: Enzyme-linked immunosorbent spot.

^aChemotherapy, radiation therapy, hormonal therapy, or cytokine therapy given/allowed at the same time as vaccine therapy

Table 5

Vaccine trials using viral vectors

Year(s) of publication + Reference Citation(s)	Type of Study	Vaccin	Type of Cancer	No. of Patients: Enrolled; Finished treatment; Control	Toxicity	Immune response	Strongest Benefit Reported ^c	Concurrent therapy ^a
<i>Viral vector vaccines</i>								
Elkord <i>et al.</i> 2009 (85); Elkord <i>et al.</i> 2008 (86)	Phase II trial	MVA-5T4 (TroVax)	To be resected colorectal liver metastases	20 (19 CRC); 16; 0	No grade III/IV toxicities	18 of 19 CRC patients had detectable 5T4 specific Ab levels following vaccination; 13 of 20 patients demonstrated vaccine-related 5T4-specific proliferative responses; 8 patients showed an increase in 5T4-specific IFN-γ ELISPOT responses after vaccination	Those with above median 5T4-specific proliferative responses showed significantly longer survival compared with those with below median responses	None
Kaufman <i>et al.</i> 2008 (74)	Randomized phase II trial (3 treatment arms: ALVAC 1) with or 2) without tetanus adjuvant before and concomitantly with chemotherapy or 3) chemotherapy followed by ALVAC)	ALVAC-CEA-B7.1	Metastatic colorectal cancer	119; 26; 0 ^b	Serious adverse events were largely gastrointestinal (n = 30) and hematologic (n = 24)	3 of 97 patients showed increase in anti-CEA antibody titers; Increases in CEA-specific T-cells (IFN-γ ELISPOT) were detected in 1) 50%, 2) 37%, and 3) 30% of patients; no differences in immune responses between the treatment groups	42 of 104 patients showed objective clinical responses (2 complete response, 40 partial response) without differences between the treatment arms	Chemotherapy (fluorouracil, leucovorin and irinotecan)

Table 5

Continued

Year(s) of publication + Reference Citation(s)	Type of Study	Vaccin	Type of Cancer	No. of Patients: Enrolled; Finished treatment; Control	Toxicity	Immune response	Strongest Benefit Reported ^c	Concurrent therapy ^a
Harrop <i>et al.</i> , 2007 (84)	Phase II trial	MVA-5T4 (TroVax)	Metastatic colorectal cancer	17; 11; 0	Safe and well tolerated with no serious adverse events attributed to TroVax	10 of 11 patients showed 5T4-specific Ab responses after vaccination; 5T4 specific IFN-γ ELISA responses detected in 10 patients	6 of 11 had complete or partial responses; 5T4 specific IFN-γ ELISA responses immune responses correlated with clinical benefit	Chemotherapy (OxMdG)
Harrop <i>et al.</i> , 2006 (83)	Phase I/II trial (dose escalating)	MVA-5T4 (TroVax)	metastatic colorectal cancer, who had responded to or stabilized on first-line chemotherapy	22; 17; 0	Safe and well tolerated with no serious adverse events attributed to TroVax	14 of 17 patients had detectable Ab levels following vaccination; in 9 of 17 patients 5T4-specific cellular proliferation responses were induced	Disease stabilization (3 to 18 months) was observed in 5 patients; a positive association between the development of a 5T4 antibody response and patient survival or time to disease progression	None

Table 5

Continued

Year(s) of publication + Reference Citation(s)	Type of Study	Vaccin	Type of Cancer	No. of Patients: Enrolled; Finished treatment; Control	Toxicity	Immune response	Strongest Benefit Reported ^c	Concurrent therapy ^a
Menon <i>et al.</i> 2003 (81); van der Burg <i>et al.</i> 2002 (82)	Phase I/II trial (dose escalation)	ALVAC-p53	Metastatic colorectal cancer	16; 15; 0	Fever was the only vaccination-related adverse event 7% patients grade 3 fever	3 of 15 showed the induction of p53-specific IgG; in 2 of 5 patients with the highest dose vaccine number of p53-specific T-cell was increased (IFN-γ ELISPOT), no p53 specific proliferation was found	1 of 15 patients showed stable disease	None
Ullenhag <i>et al.</i> 2003 (80)	Phase I trial (with or without GM-CSF)	ALVAC-Ep-CAM/KSA	Stages I-III colorectal carcinoma	12; 12; 0	The adverse reactions to the vaccinations were mild except for local skin reactions	No IgG antibodies against Ep-CAM were detected; in the ALVAC-KSA group in 2 of 6 patients, and in the ALVAC-KSA/GM-CSF group a marked IFN-γ was induced in 5 of 6 patients.	Not determined	None
Marshall <i>et al.</i> 2000 (78)	Randomized phase I trial (VAAA or AAVV)	ALVAC-CEA; rV-CEA	stage IV CEA+ malignancy without radiographic evidence of disease	21 (13 CRC); 18 (11 CRC); 0	The treatment was extremely well tolerated	CEA-specific T-cell precursor frequencies (IFN-γ ELISPOT) observed in 6 of 6 patients VAAA cohort and 2 of 5 five patients AAVV cohort	No objective anti-tumor responses	None
Horig <i>et al.</i> 2000 (75)	Phase I trial (dose escalating)	ALVAC-CEA-B7.1	CEA+ metastatic cancer	18 (10 CRC); 16 (9 CRC); 0	No evidence of significant toxicity or autoimmune reactions	4 of 16 patients showed an increase in CEA specific T-cells	3 of 16 patients experienced clinically stable disease that correlated with increasing CEA-specific precursor T-cells (ELISPOT)	None

Table 5

Continued

Year(s) of publication + Reference Citation(s)	Type of Study	Vaccin	Type of Cancer	No. of Patients: Enrolled; Finished treatment; Control	Toxicity	Immune response	Strongest Benefit Reported ^c	Concurrent therapy ^a
Von Mehren <i>et al.</i> 2000 (76)	Phase I trial (dose escalating)	ALVAC-CEA-B7.1	CEA+ advanced or metastatic cancer	39 (28 CRC); 30; 0	No evidence of significant vaccine related toxicity	2 of 31 showed the induction of CEA-specific IgG; 12 of 15 HLA-A2+ patients demonstrated increases in their CEA-specific T-cell precursor frequencies (IFN-γ ELISPOT)	6 of 30 patients had declines in CEA levels; 8 of 30 patients all had stable disease after four vaccinations	None
Zhu <i>et al.</i> 2000 (77); Marshall <i>et al.</i> 1999 (79)	Phase I trial (dose escalating)	ALVAC-CEA	stage IV CEA+ malignancy without radiographic evidence of disease	20 (13 CRC); 19; 0	no significant toxicity was attributed to the treatment	In seven of nine patients evaluated, statistically significant increases in CTL precursors specific for CEA were observed in PBMCs after vaccination, and capable to lyse allogeneic human carcinoma cell lines expressing CEA	No objective antitumor response was observed during the trial in patients with measurable disease	None

Abbreviations: AAV: three vaccinations of ALVAC-CEA followed by rV-CEA; Ab: antibody; ALVAC: recombinant canarypox virus; CEA: carcinoembryonic antigen; ELISA: Enzyme-linked immunosorbent assay; GM-CSF: granulocyte macrophage colony-stimulating factor; IFN-γ: Interferon-γ; MVA: modified vaccinia Ankara; OxMdG: oxaliplatin, folinic acid, 5-fluorouracil; rV-CEA: a live vaccinia virus; VAAA: rV-CEA followed by three vaccinations of ALVAC-CEA.

^aChemotherapy, radiation therapy, hormonal therapy, or cytokine therapy given/allowed at the same time as vaccine therapy; ^bnumbers included in the different treatment arms: (1) 39, (2) 40, (3) 39 patients

DISCUSSION

One of the most unique features of the immune system consists of its capacity to specifically search and destroy targets. As such, many have discussed if tumor cells represent one of the regular targets of the immune system and in addition if the patient's own immune system can be used to specifically destroy tumor cells once tumor cells escaped immune surveillance (10). Subsequently, many have tried to reinforce the immune system to cure cancer patients. In this review we provided an overview of currently tested vaccines to treat colorectal cancer patients. The few trials focused on clinical efficacy comprised phase III trials using irradiated tumor samples which suggested some clinical benefit in selected subpopulations (25,29) but overall were rather disappointing. However, most of the trials have been designed to test the safety and immunogenicity of vaccines but have yet not resulted in the design and execution of phase III trials. Although in most trials no serious vaccine related adverse events were noted, lack of clinical results suggests that the vaccine-induced T-cell responses against these antigens are at this point not robust enough or of sufficient quality to confidently progress to efficacy trials. In general, a stronger focus should be put on how to induce the strongest and best qualified leukocyte population by vaccination. Vaccines should be combined with adjuvants to induce a vaccine specific Th1 polarized response and suppress a Th2 response. At the moment many candidate adjuvants, including monophosphoryl Lipid A, poly I:C, CpG and different cytokines are available. Also chemotherapeutics and monoclonal antibodies comprise strong immune modulating agents that can be used to polarize a response after vaccination. Various mechanisms may explain the reported synergistic effects of chemotherapy and immunotherapy. Direct effects of chemotherapy on tumor or host environment, such as induction of tumor cell death, elimination of regulatory T cells, and/or enhancement of tumor cell sensitivity to lysis by CTL may account for enhancement of immunotherapy by chemotherapy. On the other hand, immunotherapy may directly modulate the tumor's sensitivity to chemotherapy (87). Indeed, results have suggested that MVA-5T4 vaccination can be combined with chemotherapy (84). Recently, the CTLA-4 blocking antibody ipilimumab has been successfully used in the treatment of melanoma patients (17). Also other monoclonal antibodies such as anti-PD-1 antibodies will become available in the near future. As single agent, anti-PD-1 antibodies may occasionally provide clinical benefit (88) Based on their mechanisms of action it is highly likely that these antibodies will synergize with strong vaccines as they will block the negative feed-back on vaccine-induced tumor-specific T cells.

The most recent vaccine developments suggest that some of the current vaccine strategies do harbor the capacity to induce strong immune responses in cancer patients even to self-antigens. In particular, we and others have found that the use of long overlapping peptides allow the activation of strong and broad T-cell immunity to HPV, p53, NY-ESO and HER2/neu (63,89,49,90-94). While these vaccines may still have to be optimized, the data suggest that the vaccine-induced

activation of tumor-specific T-cell reactivity is no longer an issue of concern. However, other relevant questions remain:

- What are the tumor antigens recognized by tumor-infiltrating T-cells, and which antigens would be most appropriate in colorectal cancer?
- Does vaccination only enhance effector T-cells or also suppressive T-cells?
- Do vaccine-induced tumor-specific leukocytes migrate to the tumor and mediate an antitumor effect?
- Which cancer patients are most likely to benefit from immunotherapy?

Frame-shift mutated gene product-derived peptides, a class of tumor-specific antigens

Despite many years of work, the number of antigens recognized by TILs of colorectal cancer identified is limited (95-98). Consequently, vaccines so far have been developed on the basis of proteins that are selectively expressed by tumor cells but for which immunity can be blunted, such as p53 where the CD8 T-cell component suffers from central tolerance, or may lead to autoimmunity such as has been observed with CEA (48,50). The exception comprises microsatellite instable (MSI-H) tumors that, due to numerous of frameshift mutations in microsatellites express neo-antigens. MSI-H is a molecular feature of tumors associated with the familial Lynch or hereditary non-polyposis colorectal cancer (HNPCC) syndrome, accounting for approximately 5% of all colorectal cancer cases (99-101) and for approximately 15% of all sporadic colorectal, gastric and endometrial cancers, as well as at lower frequencies for various other sporadic cancers (102-106). MSI-H colorectal tumors are predominantly localized in the proximal colon, comprising 50% of all proximal colon tumors (107,108). Since frameshift mutated products (FSPs) are foreign to the immune system, they represent a unique group of tumor-specific antigens. No tolerance and consequently strong T-cell responses are expected against these FSPs. Unfortunately, relatively little is known on the immunogenic behavior of such FSPs (98). A few studies have been performed to predict the immunogenic behavior of a selection of frame-shift mutated genes which are frequently detected in MSI-H cancers (98,109,110). We developed a methodology for predicting their immunogenic behavior that is based on accumulation and MHC class I presentation (109). Our data indicated that, out of the 15 FSPs examined in our study, 4 (TGF β R2-1, MARCKS-1, MARCKS-2 and CDX2-2) are of primary interest (109). Four additional antigens (TAF1B-1, PCNXL2-2, TCF7L2-2 and Bax α +1) are of moderate interest for further tumor immunological research (109). The data of others suggested that FSP-specific T-cells may be present in the circulation of patients with MSI-H colorectal cancer, healthy HNPCC syndrome mutation carriers, but not in patients with microsatellite stable (MSS) colorectal cancer or in healthy donors (110,111). In general, most FSPs consist of a relatively small number of amino acids downstream of the frame-shift mutation, suggesting that the FSPs may contain a sequence that can only be presented by a limited number of HLA class I or HLA class II molecules. In order to treat patients, knowledge on which HLA class I and II molecules can present epitopes comprised by the FSPs should

be obtained. Although MSI-H tumors comprise only about 15% of all colorectal tumors, patients with a MSI-H tumor are very interesting vaccination candidates because: 1) strong effector responses are expected after vaccination using non-self-antigens; 2) colorectal cancer is one of the major cancers in the western world; and 3) many families with Lynch or HNPCC syndrome at risk for a MSI-H tumor have been identified. The latter group may be amenable for prophylactic vaccination to prevent the outgrowth of MSI-H tumors. Hence, a rapid identification of the immunogenic non-self-segment of the frame-shift products is required. Notably, the amino acid length of the frame-shift products make them perfect candidates for overlapping synthetic long peptide-based vaccines that have been shown to be highly immunogenic in rodents and human beings as we discussed above (56).

Does vaccination only enhance effector T-cells or also suppressive T-cells?

In contrast to animal models, the history of constant interactions between tumor and immune system shapes both tumor and the immune system of an individual patient in a way that is difficult to mimic in animal tumor models. It is of utmost importance that vaccines only boost the reactivity of immune cells that mediate an antitumor effect and not that of immune cells that support tumor growth. Therefore, to gain a thorough understanding of the immunological events occurring in patients in vaccination trials it is in our opinion crucial to comprehensively perform immune monitoring during vaccination trials. The results from immune monitoring make it possible to understand possible clinical effects, to guide the optimization of vaccination strategies and may even encourage investigators to move the product forward into the clinic (24). Unfortunately, most immunotherapeutic vaccine trials mostly report on one particular aspect of the desired immune response (e.g. HLA-multimer+ cells, IFN- γ -producing cells). They do not include more detailed analyses of the total vaccine-modulated immune response. Colorectal cancers are infiltrated by both CD4⁺ and CD8⁺ Foxp3⁺ Tregs, and the number of Foxp3⁺ Tregs negatively correlates with disease stage and survival in colorectal cancer (112-114). Notably, the analyses of the antigens recognized by colorectal cancer infiltrating Tregs revealed that they recognized colorectal cancer-associated antigens, in particular Mucin, Her-2/neu, and CEA (96). Hence, therapeutic vaccination with these antigens may not only boost CD4⁺ and CD8⁺ effector T-cells but also the Treg population. Vaccine-induced expansion of such antigen-specific Tregs has been observed previously in a mouse tumor model (115) and also in humans (63). More specifically, in this study the magnitude of the vaccine-enhanced antigen-specific Treg response was related to clinical failure of an otherwise successful therapeutic vaccine for premalignant disease (116). In a trial conducted by our group, in which colorectal cancer patients were vaccinated with overlapping p53 long peptides, strong p53-specific CD4⁺ T-cell responses were found but this did not coincide with the expansion of p53-specific CD4⁺Foxp3⁺ T-cells (49). This fits with the observation that the spontaneous T-cell response to p53 in colorectal cancer patients is not under control of Tregs (96). In human beings several approaches have been used to delete Tregs (117). Notably, decreases in CD4⁺CD25⁺Foxp3⁺ cells have

been detected when patients with hepatocellular cancer were treated with low cyclophosphamide (118), as well as in metastatic melanoma patients treated with the anti-CD25 antibody Daclizumab (119), or after using denileukin diftitox (120). Whereas, the use of Daclizumab did not enhance the efficacy of the peptide-pulsed DC vaccine (119), multiple injections of denileukin diftitox did result in enhanced CEA-specific T-cell responses (120).

Migration of tumor-specific T-cells

The success of immune responses partly depends on the ability of effector cells to infiltrate the cancer microenvironment and finally contact and attack tumor cells. As most TAA are intracellular proteins and results from observational studies show that especially presence of intra-epithelial activated CD8⁺ T-cells has a positive impact on prognosis (13), vaccine strategies should focus on the induction of tumor-specific CD8⁺ T-cell responses. The function of cytotoxic T-cells depends on a network of collaborating leukocytes. Consequently vaccines should create a CD8⁺ T-cell friendly and supportive cancer microenvironment. Data from other studies in colorectal cancer indicate that especially tumor-specific CD4⁺ Th1 cells are associated with a supportive cancer microenvironment that is beneficial to the prognosis of cancer patients (121-123). It has been well documented that CD4⁺ T-cells not only license the priming of tumor-specific CD8⁺ T-cells but are important to sustain their fitness (56). Moreover, tumor-specific CD4⁺ Th1 cells are needed for the production of IFN- γ in order to modulate the local tumor microenvironment in such a way that it will produce chemokines that attract CD8⁺ T-cells (124,125). In addition, the IL-2 produced by these CD4⁺ Th1 cells enhances local CD8⁺ T-cell proliferation and cytolytic function (125). Indeed chemokine expression as well as that of endothelial adhesion molecules and extracellular matrix has been associated with the migration of leukocytes into colorectal carcinoma (126-129). Vaccines can induce tumor-specific Th1 cells but they may make up only a minor population of all tumor-specific T-cells that are induced by therapeutic vaccines. In our series about 60% of the patients vaccinated with the p53 SLP vaccine mounted p53-specific CD4⁺ T-cells that were able to produce IFN- γ as measured by IFN- γ ELISPOT (49). Assessment of all p53-activated CD4⁺ T-cells, by gating on the CD4⁺CD154⁺ T-cell population by multiparameter flow cytometry, however, revealed that the IFN- γ -producing population of T-cells was only in a minority of cases the major subset among the vaccine-induced p53-specific T-cells (49) suggesting that new trials should focus on improving the percentage of p53-SLP vaccine-induced Th1 polarized T-cells in order to benefit from the local effect of tumor-specific Th1 cells.

Optimization of vaccination studies results in clinical success

Although many studies determined the induced immune response after immunization, no gold standard has been set to define clinical response after vaccination (130). Activation of the cellular arm of the immune system is seen as the first biological event after administration of immunotherapy, consequently determination of the immune response is of great interest. Many different bioassays have

been developed for immune monitoring: enzyme-linked immunosorbent spot (ELISA), carboxyfluorescein succinimidyl ester-based proliferative assays, HLA peptide multimer staining and flow cytometry-based tests. Unfortunately substantial variability in results among laboratories prohibits data reproducibility and prevents meaningful comparison among studies. Therefore initiatives have been put up to standardize immune monitoring and harmonize cellular immune assays. Harmonization will establish the use T-cell-based assays as a reproducible gold standard for immunotherapy and reliable parameter to determine the correlation between induced T-cell responses and clinical events. Under the auspices of the CIMT Immunoguiding Program (CIP), a working group founded under the aegis of the Association for Cancer Immunotherapy (CIMT) and the Cancer Immunotherapy Consortium under the auspices of the Cancer Research Institute (CRI) two large immune monitoring programs were initiated to provide a quality assurance process for laboratories conducting immune monitoring and harmonize assay performance (131-133).

An important question that remains is which cancer patients are best candidates to study clinical endpoints once safety and immunogenicity of a therapeutic vaccine strategy have been established. So far most trials have included end-stage patients only. Although regression of tumor mass can be very convincing and objectively measured, vaccination of end-stage patients may present with several drawbacks that negatively influence the immunotherapeutic effect. Major drawbacks are the suppressed immune status, the general short survival period that may obscure clinical effects of therapy at later time points (134), a large immunosuppressive tumor mass, variety of treatments before vaccination, and co-morbidity. Therefore clinical endpoints might be best studied in an adjuvant rather than an end-stage setting. These patients, who have no measurable tumor mass and a relatively normal functioning immune system are expected to respond optimally to immunization. Classically, the clinical effects of therapeutic vaccines have been determined shortly after vaccination by Response Evaluation Criteria In Solid Tumors (RECIST) (130,135,136). These criteria depend on tumor shrinkage and were initially optimized for chemotherapeutic agents (130,135,136). Immunotherapeutics often demonstrate delayed clinical responses resulting in a delayed separation of Kaplan-Meier curves. To compensate delayed separation in statistical models altered statistical models describing hazard ratios as a function of time and recognizing differences before and after separation of curves may allow improved planning of phase III trials (130). Especially for phase III trials if therapeutic vaccines are used in an adjuvant setting and consequently patients lack presence of tumor mass. Furthermore, the differences in immune-induced clinical response patterns when compared to those observed after treatment with cytotoxic agents (130), have led to the use of new immune-related response criteria instead of RECIST to more comprehensively capture all response patterns over weeks to months (134,130,137).

EXPERT COMMENTARY

For many years vaccine strategies have failed to induce strong and broad immune responses in patients with cancer. New vaccine strategies and platforms have resulted in vaccines that are more powerful but still require optimization. In addition, vaccine strategies in colorectal cancer still suffer from a lack of antigens that may be used for vaccination. Whereas for other types of tumors the reactivity of tumor-infiltrating T-cells validate the choice of antigen used in the vaccines for that type of cancer, this is still limited in colorectal cancer and calls for more in-depth studies on the specificity of T-cells infiltrating the tumor or present in metastatic lymph nodes. In view of the increasing role of CD4⁺ T-cell help to the induction, sustenance and migration of CD8⁺ T-cells, it is advisable to screen not only for CD8⁺ T-cell responses but also for tumor-specific CD4⁺ T cells. The absence of clearly defined antigens applicable to a majority of colorectal cancer patients may have provided the rationale to use tumor cell-derived vaccines to be tested in phase III trials. These have all failed to induce a statistically significant positive clinical reaction. The failure to show clinical efficacy may have been the result of delayed clinical reactivity also seen with other immunotherapeutic approaches but is more likely to reflect poor immunogenicity. Due to the absence of defined clinically important antigens it will be hard to perform studies showing that immunogenicity of these types of vaccines can be improved. Unfortunately, defined tumor antigen-based vaccines have not been tested in a phase III study. Their design, however, will not only provide the opportunity to fully optimize their activity under all sorts of conditions, but also allows studying their effects locally within the tumor. Still such vaccines may be unsuccessful due to all sorts of reasons that play a role in colorectal cancer, including the fact that colorectal cancer can evolve via several different routes. To obtain proof-of-concept, the immunotherapy of colorectal cancer may want to concentrate on the treatment of tumors with microsatellite instability as they are known to be heavily infiltrated by T cells and express tumor-specific antigens that are derived from frameshift-mutated gene products.

FIVE-YEAR VIEW

There is a clear role for tumor-specific T-cell immunity in the final clinical outcome of colorectal cancer. Hence, a continued effort will be put in the development of vaccines and vaccine strategies against colorectal cancer. Despite that some of the current vaccines are able to induce strong antigen-specific immune responses in the absence of serious adverse events, there is hardly any evidence generated to show the clinical impact of these vaccines in patients with colorectal cancer. The current successes in the treatment of cancer by activation of the host's immune system has spirited studies aiming to improve the clinical impact of colorectal cancer vaccines. It is not likely that colorectal cancer vaccines are able to induce the desired clinical responses on their own, but need to be combined with other

Key issues in the development of anti-cancer-vaccines for colorectal cancer patients:

- Vaccines used in clinical trials to treat colorectal cancer patients are safe and immunogenic.
 - The vaccines used lack clinical effect or have not been tested for efficacy.
 - T-cell based immunotherapy has been shown to mediate clinical effects in other cancer types.
 - To obtain clinical results in colorectal cancer vaccine strategies may require to:
 - Select truly tumor-specific antigens;
 - Enhance the number of effector T cells but not that of suppressive T cells;
 - Create a T cell friendly and supportive cancer microenvironment;
 - Combine vaccination with other immune stimulating treatment modalities;
 - Vaccinate patients in the adjuvant setting and not end-stage patients;
 - Harmonize immune monitoring to allow comparison of trial data and to boost the development of working strategies.
 - Persons with MSI-H colon cancer or at risk for MSI-H tumors comprise a unique opportunity for respectively therapeutic or prophylactic vaccination using non-self frameshift-mutated peptides.
-

modalities that target regulatory mechanisms in order to improve the local micro-environment. The current wealth of preclinical and clinical information predicts a future strategy in which therapeutic vaccines, blockers of immunosuppressive mechanisms and conventional therapies are applied jointly to overcome immunological tolerance and promote tumor regression. It will not be trivial to synchronize all the different treatment modalities but help may come from the generation of immunological data sets analysing the supposed mechanism of action as well as that of non-desired immune reactions expected to occur by the treatment modalities. As most trials in colorectal cancer are currently performed by small biotechnology companies or even academic institutes, we will see that they will harmonize their immune monitoring efforts in order to fully profit from the results generated by vaccination strategies that use different additional treatment modalities. We anticipate and endorse that most of the financial power will flow into phase I/II trials synchronizing a multimodality treatment to enforce local antitumor reactivity. This means that the move from phase I/II trials to phase III trials, which are a prerequisite to establish efficacy of T-cell mediated immunotherapy for colorectal cancer patients is not likely to happen soon, but may follow a few years from now.

GRANT SUPPORT:

Part of this study was defrayed from The Netherlands Organization for Health Research and Development (project no. 92003509 to FMS).

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CHAPTER 7

Addition of interferon-alpha to the p53-SLP[®] vaccine results in increased production of interferon-gamma in vaccinated colorectal cancer patients: A phase I/II clinical trial

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International Journal of Cancer. 2012; 132 1581-1591

ABSTRACT

Purpose: We previously established safety and immunogenicity of a p53 synthetic long peptides (p53-SLP®) vaccine. In the current trial we investigated whether combination of Interferon-alpha (IFN-α) with p53-SLP® is both safe and able to improve the induced p53-specific IFN-γ response.

Experimental design: Eleven colorectal cancer patients successfully treated for metastatic disease were enrolled in this study. Of these, nine patients completed follow up after two injections with p53-SLP® together with IFN-α. Safety and p53-specific immune responses were determined before and after vaccination. Furthermore, cryopreserved PBMCs were compared head-to-head to cryopreserved PBMCs obtained in our previous trial with p53-SLP® only.

Results: Toxicity of p53-SLP® vaccination in combination with IFN-α was limited to grade 1 or 2, with predominantly small ongoing swellings at the vaccination site. All patients harbored p53-specific T cells after vaccination and most patients showed p53-specific antibodies. Compared to the previous trial, addition of IFN-α significantly improved the frequency of p53-specific T cells in IFN-γ ELISPOT. Moreover, in this trial, p53-specific T cells were detectable in blood samples of all patients in a direct *ex vivo* multiparameter flowcytometric assay, opposed to only 2 out of 10 patients vaccinated with p53-SLP® only. Finally, patients in this trial displayed a broader p53-specific immunoglobulin-G response, indicating an overall better p53-specific T-helper response.

Conclusions: Our study shows that p53-SLP® vaccination combined with IFN-α injection is safe and capable of inducing p53-specific immunity. When compared to a similar trial with p53-SLP® vaccination alone the combination was found to induce significantly more IFN-γ producing p53-specific T-cells.

INTRODUCTION

The modest to poor prognosis of colorectal cancer patients treated with curative intent, calls for additional treatment modalities such as immunotherapy (1).

p53 is one of the most frequently used tumor-associated antigens in tumor directed vaccination studies (2). Due to a mutation, p53 is inactivated and over-expressed in 34-45% of colorectal tumors, while wild-type p53 is expressed at extremely low levels (3). This provides an appropriate immunological window for T cells, being targeted to p53, to discriminate between tumor cells and normal cells (4).

A clinical-grade p53 synthetic long peptides (p53-SLP®) vaccine was developed that was tested in two parallel phase I/II studies in colorectal and ovarian cancer patients (5-7). Results from these first trials revealed that in the vast majority of vaccinated cancer patients mainly p53-specific CD4⁺ T cells were induced (6,7).

The presence of tumor-specific CD4⁺ T cells in the cancer microenvironment is a prerequisite for support, proliferation, recruitment and cytolytic function of tumor-specific CD8⁺ T cells, greatly accelerated by the production of IFN- γ and IL-2 (8-10). Patients with metastatic colorectal cancer vaccinated against the tumor antigen 5T4 were found to have more clinical benefits when 5T4-specific IFN- γ ELISPOT responses were induced (11). Also, in women with human papillomavirus (HPV) positive vulvar intraepithelial neoplasia, complete responses after vaccination against HPV were positively associated with the induction of IFN- γ -producing and proliferative T-cell responses (12). Together, these data suggest that clinical responses after vaccination depend on the induction of strong and broad vaccine-specific type 1 T-cell responses. Results from the first two trials with p53-SLP® showed that vaccine-induced type 1 T-helper (Th1) cells produced only low amounts of the key cytokines (i.e. IFN- γ and IL-2), indicating that tumor-induced p53-specific Th-responses are present but not properly polarized (6,7). Therefore, in order to benefit from the tumor-specific Th cells at the tumor site, the p53-SLP® should be combined with immune modulating adjuvants that specifically induce Th1-cell polarization. A possible candidate adjuvant to achieve this is Interferon-alpha (IFN- α).

IFN- α is used to treat patients suffering from chronic viral hepatitis infection and different malignancies (13). IFN- α plays a major role in the differentiation of the Th1 subset, the generation of CTL and the promotion of proliferation and survival of T cells (14,15). Moreover, several studies have shown that type I IFNs promote the differentiation of monocytes into dendritic cells (DC) and enhance DC activity (16-21). In a murine melanoma model, it was shown that addition of IFN- α to a gp100 peptide, suppressed melanoma growth and increased the accumulation and proliferation of gp100-specific, IFN- γ -secreting CD8⁺ T cells (22). Moreover, adoptive transfer of tumor-reactive T cells and daily injections of IFN- α in metastatic melanoma patients can lead to successful treatment of metastatic melanoma (23). In humans, peptide vaccination has been combined with IFN- α injections showing that the combination was safe, resulted in a consistent enhancement of vaccine-specific CD8⁺ T cells and increased the percentage of blood circulating DC

precursors (24). We now report the results of a phase I/II trial addressing safety and immunogenicity in which successfully treated metastatic colorectal patients were subcutaneously vaccinated with p53-SLP[®] in combination with subcutaneous administration of IFN- α . In addition, we analyzed whether addition of IFN- α close to the vaccine site not only induced a stronger p53-specific but also a better polarized Th1 response by testing and comparing cryopreserved peripheral blood mononuclear cells (PBMCs) and serum samples of the current trial head-to-head to samples obtained in our previous clinical trial, in which a similar group of colorectal cancer patients were vaccinated with the p53-SLP[®] vaccine only (6).

PATIENTS, MATERIALS AND METHODS

Patients, vaccination scheme and safety and tolerability monitoring

Colorectal cancer patients who were successfully treated with metastasectomy, chemotherapy and/or Radiofrequency Ablation (RFA) for disease metastasis to the liver and/or the lung were accrued during their follow up visits at the surgical oncology out-patient clinic into this phase I/II trial. Primary endpoint of this study was safety and immunogenicity of the p53-SLP[®] in combination with administration of IFN- α . The secondary endpoint was to assess whether this combination is able to induce an overall significantly stronger p53-specific Th1 response than observed in the group of patients vaccinated in our previous trial (6). Patient eligibility criteria for in- and exclusion and the study design, including the vaccination schedule (twice vaccinated with a three week interval), were identical to those used in the previously performed clinical trial with p53-SLP[®] (6), with the exception that in the current study one hour after each vaccination pegylated interferon-alpha-2b (Pegintron, 1 μ g/kg body weight, Schering-Plough, the Netherlands) was injected within 10 centimeters proximity to the vaccination site. Furthermore, patients were discharged within one hour after they received their Pegintron injection. The study design was approved by the Central Committee on Research Involving Human Subjects in The Hague, the Netherlands (NL24089.000.08) and by the medical ethical committee of the Leiden University Medical Center. All patients gave their written informed consent before they were enrolled in the study.

Patients were asked to monitor and report any adverse event (AE) including fever (temperature measured at home either orally or anally above 38°C). Prompted and spontaneous AEs, injection site reactions, clinical assessments, and clinical laboratory variables were monitored during all visits as reported previously (25). Injection site reactions were defined as pain, redness, itch and calor on a scale of 0-3 (0 being no reaction, 1 as mild, 2 as moderate and 3 as a severe reaction). Local swelling was measured bi-directionally in cm. Before each vaccination the medical history was taken and blood was drawn (both for safety and immunological assessment). In addition, the patients were physically examined before and after each vaccination.

Table 1

Patient characteristics of patients enrolled

Patient Number	Sex	Age	TNM	Location Primary	Location Metastasis/ Recurrence	Treatment	Clinical status (months NED)	P53 status
p20	M	62	4	Ascending colon	Liver	PR/CTx/Rli RFA	Alive (15)	3
p21	M	44	1	Rectum	Liver,LR	PR/RTx/CTx/Rlu/ CTxLu	Alive (14)	2
p22*	F	57	4	Sigmoid colon	Liver	PR/CTx/Rli	Withdrew consent	X
p23	M	60	4	Rectum	Lung	PR/RTx/CTx/Rlu/ CTxlu/RFA	Alive (2)	3
p24	F	61	3	Rectum	Liver	PR/RTx/Rli/CTx/ RFA	Alive (12)	2
p25	F	50	4	Sigmoid colon	Liver	PR/Rli/CTx/RFA	Alive (12)	X
p26	M	52	4	Rectum	Liver	PR/Rli/CTx	Alive (2)	3
p27	M	60	4	Cecum	Liver	PR/Rli/CTx/CTxli/ RFA	Death (5)	1
p28*	M	64	4	Rectum	Liver	PR/RTx/CTx	Withdrew consent	X
p29	M	65	4	Rectum	Liver	PR/RTx/CTxli/ RFA/Rli	Alive (7)	1
p30	F	64	3	Ascending Colon	Liver	PR/CTx/Rli	Alive (4)	2

Abbreviations: TNM: Tumor Node Metastasis Stage at time of diagnosis primary tumor. F; female. M; male. Lr: local recurrence. RTx; neo-adjuvant radiotherapy, Pr; primary resection. CTx; (neo) adjuvant chemotherapy. CTxlu; isolated lung perfusion, CTxli: isolated liver perfusion, Rlu; resection lung lesion. Rli; resection liver lesion. RFA: radiofrequency ablation. NED; no evidence of disease (months between second vaccination and disease recurrence or last follow up date). P53 status immunohistochemistry (IHC). 1: expression of p53 in <25% of the tumor cells; 2: expression of p53 in ≥25% but <75% of the tumor cells; 3: expression of p53 in ≥75% of the tumor cells; X: no material was available for IHC. *Patient number 22 withdrew consent after the first vaccination due to adverse event (table 2). Patient number 28 withdrew consent before the first vaccination.

Vaccine

The clinical-grade peptides (9 peptides of 20-30 amino acids long with an overlap of 5-14 amino acids) were synthesized at the Department of Clinical Pharmacy and Toxicology, Leiden University Medical Center (LUMC), the Netherlands, together representing the part of the p53 protein from amino acid positions 70 to 235. In comparison to our previous trial one long peptide (i.e. the peptide with amino acid sequence 224-248) was not included in the current vaccine mixture, because yields of the synthesis and purification of this peptide were very low. At the day of vaccination, the vaccine was prepared as previously described (6).

Immunohistochemistry and evaluation

The expression of p53 by colorectal tumor cells was determined in the available paraffin-embedded metastatic tissue of the vaccinated patients by standard two-step indirect immunohistochemical staining as described previously (6). The percentage of tumor cells expressing p53 (nuclear expression), together with internal control, was estimated and categorized into three groups: (1) expression of p53 in <25% of the tumor cells; (2) expression of p53 in $\geq 25\%$ but <75% of the tumor cells; (3) expression of p53 in $\geq 75\%$ of the tumor cells.

P53-peptide ELISA for IgG antibodies

Serum samples (pre-vaccination and 3 weeks after the second vaccination) of the colorectal cancer patients from both trials were subjected to a p53-peptide ELISA for detection of p53 peptide-specific immunoglobulin G (IgG). A 96-wells plate (Costar 3590) was coated overnight at 4°C with the individual p53 peptides (30-mers, 14 amino acids overlap; 50 μ l of 1 μ g/ml diluted in 0.1M carbonate/bicarbonate coating buffer; Merck, Darmstadt, Germany). Then, the plate was washed 6 times with phosphate buffered saline (PBS; Fresenius Kabi Bad Homburg, Germany) +0.05% Tween (Merck) and blocked for 1 hour at room temperature (RT) in 100 μ l/well PBS+0.05% Tween+0.1% bovine serum albumin (BSA; Sigma Aldrich, St Louis, MO, USA), which is assigned as blocking buffer. After 6 washings with PBS+0.05% Tween, the serum samples diluted in blocking buffer (1:100) were added to triplicate wells (50 μ l/well) and incubated at RT for 2 hours. The plate was washed again and 50 μ l/well of goat anti-human IgG-Horseradish peroxidase (HRP; Southern Biotechnology, Birmingham, AL, USA) (diluted 1:3000 in blocking buffer) was added and incubated for 1 hour at RT. Finally, after the 6 washings tetramethyl-benzidine liquid substrate (50 μ l/well TMB, Sigma Aldrich) was added for the colorimetric enzymatic reaction. This reaction was stopped by adding 50 μ l/well of 2M H₂SO₄ (Merck) and the plate was read in an ELISA reader at 450 nm. A cut-off value was calculated to define a positive response. For this the average OD-value plus 2xSD of the triplicate wells for all 12 peptides per plate in a pre- or post-vaccination serum sample was calculated. All OD-values above this cut-off value were discarded and again the average+2xSD was calculated with the remaining OD-values. This process was repeated until all OD-values were below the last calculated cut-off value. At least 2 of the 3 OD-values per triplicate peptide test needs to be above this cut-off value, then a peptide was considered to yield a positive response, i.e. is recognized by specific IgG in the serum. A 2-fold increase of the post-vaccination serum sample over that of the pre-vaccination serum sample was considered a vaccine-induced positive response and calculated as fold induction. The average number of vaccine-induced positive responses for the individual peptides was determined in the group of patients from both clinical trials. Subsequently, the number of positive peptide reactions per patient and an overall response rate (the number of positive peptides divided by the total number of tested peptides) was calculated for both groups.

We acknowledge the concept of the Minimal Information About T-cell Assays (MIATA) reporting framework for human T-cell assays (26,27).

Cell samples

Hundred mL of heparine blood was drawn prior to vaccination and 3 weeks after the second vaccination. PBMCs were isolated using Ficoll density gradient centrifugation within 2 hours, washed with PBS, resuspended in cold Fetal Calf Serum (FCS; PAA Laboratories, Pasching, Austria) and cooled on ice for 15 minutes. After drop-wise addition in a 1:1 ratio of freezing medium (80% FCS and 20% DMSO (Sigma Aldrich)), the PBMCs were cryopreserved at 10 million per ml per vial using an automated controlled rate freezer (Cryosolutions, 's Hertogenbosch, The Netherlands), and stored in equal aliquots in a vapor phase liquid nitrogen vessel until use. The handling and storage of the PBMCs were done according to the standard operation procedures (SOPs) of the department of Oncology at the LUMC by trained personnel.

Antigens

Overlapping peptides (30-mers with 14 amino acids overlap) covering the entire p53 protein were synthesized at the department of Clinical Pharmacy and Toxicology, LUMC, with >95% purity (28), dissolved in DMSO at 50 mg/mL and further diluted in PBS to obtain a concentration 0.5 mg/mL (in PBS/1% DMSO). The clinical-grade peptides of the vaccine were used in the immune monitoring assays. PHA (HA16; Murex BioTech, Kent, UK) and memory response mix (25,29) was taken along as a positive control.

T-cell assays and data acquisition

The PBMCs were tested for p53-specificity by a set of complementary T-cell immune monitoring assays including: IFN- γ ELISPOT, lymphocyte stimulation test (LST) and cytometric bead array (CBA), all as previously described (6). Fresh PBMCs and T cells cultured out of the vaccination site biopsy were also subjected to the directly *ex vivo* intracellular cytokine staining (ICS) and analyzed as previously described (6). In this study the cells were stained for the following markers: CD3, CD4, CD8, CD154, CD137, IL-2 and IFN- γ (6,30). For a fair comparison with the results of our previous trial with metastasized colorectal patients vaccinated with p53-SLP[®] only, cryopreserved PBMCs from both trials were thawed and subjected to our novel ICS assay (25) under the same conditions. As higher concentrations of the peptides (i.e. 50 μ g/mL) were required in this new ICS assay the non-clinical grade peptides covering the complete p53 protein were used.

Data analysis and interpretation

A positive response is predefined per assay and described previously (6). For all T-cell assays, a vaccine-induced response was defined as at least a 3-fold increase in the response after vaccination when compared to the results before vaccination. Statistical analyses were conducted in SPSS (version 17.0 for Windows; SPSS, Inc).

The Fisher's exact test or the Mann-Whitney test were used to evaluate differences in patient characteristics between patients included in the current and the previous trial with the p53-SLP[®] vaccine. The Mann-Whitney test was also used to evaluate the difference in number of IFN- γ -producing T cells, the level of IFN- γ production and to compare the difference in antibody responses between the two study cohorts.

Laboratory environment

The immunomonitoring assays were performed in the laboratory of the department of Clinical Oncology (LUMC) that operates under research conditions, following SOPs and using trained staff. This laboratory has participated in all proficiency panels of the CIMT Immunoguiding Program (CIP) (<http://www.cimt.eu/workgroups/cip/>), as well as in IFN- γ ELISPOT panels of the Cancer Immunotherapy Consortium (31,32), to validate its SOPs.

RESULTS

Patient characteristics

Eleven colorectal cancer patients were enrolled in this study, 9 of whom completed all follow-up visits. The clinicopathological characteristics are displayed in table 1. None of these patients showed evidence of any macroscopic disease at enrollment. Six out of 9 patients were male. The average age of the 9 patients vaccinated twice, was 58 years. Over-expression of p53 in the tumor (i.e. $\geq 25\%$ of the tumor cells express p53) was found in 6 patients, while normal p53 expression (i.e. in $< 25\%$ of the tumor cells) was observed in 2 patients (p27, p29). Of 3 patients, the p53 status was not determined because no tumor material was available (p25) or because they prematurely withdrew consent (p22, p28).

Safety of the vaccine

The AEs of all vaccinated patients are summarized in table 2. All patients reported swelling confined to the vaccination site. In the majority of the cases the induration occurred after both vaccinations and was still present at the final check-up visit around 28 weeks after the first vaccination. The average size of these swellings at the first vaccination site was 3.3 cm and at the second site 3.5 cm. Four patients reported fever post vaccination, but it never lasted longer than 1 day. One patient also suffered from flu-like symptoms after both vaccinations for one day. Only two patients reported pain at the vaccination sites. None of the patients reported any pain, swelling or other changes of the skin at the IFN- α injection site. Only patient p22 experienced an AE exceeding grade I toxicity based on the Common Terminology Criteria (CTC) for AE version 4.0. This patient already experienced pain before vaccination in her left arm. After first vaccination, she experienced local swelling classified as an AE grade 1 and pain throughout her entire left arm, classified as an AE grade 2 and she chose to withdraw consent.

Table 2

Adverse events of patients enrolled

Patient	AE description	Relation	Action	CTC	Type	Duration
p20	Swelling at site 1	Definitely	No	Grade I	1	Ongoing
	Swelling at site 2	Definitely	No	Grade I	1	Ongoing
p21	Swelling at site 1	Definitely	No	Grade I	1	Ongoing
	Swelling at site 2	Definitely	No	Grade I	1	Ongoing
	Pain at both site 1,2	Possibly	No	Grade I	1	Ongoing
	Flulike symptoms post both vaccinations, no fever	Probably	No	Grade I	2	1 day
p22	Swelling at site 1	Definitely	No	Grade I	1	Ongoing
	Pain at site 1 and trough out left arm	Possibly	Yes*	Grade II	4	Ongoing
p23	Swelling at site 1	Definitely	No	Grade I	1	Ongoing
	Swelling at site 2	Definitely	No	Grade I	1	Ongoing
p24	Swelling at site 1	Definitely	No	Grade I	1	1,5 months
	Fever after vaccination 1	Possibly	No	Grade I	2	1 day
	Swelling at site 2	Definitely	No	Grade I	1	Ongoing
p25	Swelling at site 1	Definitely	No	Grade I	1	Ongoing
	Swelling at site 2	Definitely	No	Grade I	1	3 weeks
p26	Swelling at site 1	Definitely	No	Grade I	1	Ongoing
	Fever after vaccination 1	Possibly	No	Grade I	2	2 days
	Swelling at site 2	Definitely	No	Grade I	1	2 months
p27	Swelling at site 1	Definitely	No	Grade I	1	1,5month
	Fever after vaccination 1	Possibly	No	Grade I	2	1 day
	Swelling at site 2	Definitely	No	Grade I	1	Ongoing
p29	Swelling at site 1#	Probably	No	Grade I	1	Ongoing
	Swelling at site 2	Definitely	No	Grade I	1	Ongoing
P30	Swelling site 1	Definitely	No	Grade I	1	Ongoing
	Swelling at site 2	Definitely	No	Grade I	1	Ongoing
	Fever after vaccination 2	Possibly	No	Grade I	2	1 day

Table 2 reports all adverse events (AE) recorded for each patient included in this trial during the entire follow up period. AE were detected either at site 1 (the site of the first vaccination) or at site 2 (the site of the second vaccination). No AEs were reported with respect to the injection site of either the first or the second IFN- α administration. The heading *AE* provides a description of all the AE reported on in each patient. For each EA it is stated whether there was a plausible relation of the AE to the vaccination and whether the AE required any *actions* of the trial coordinator. All AEs were graded according to the Common Terminology Criteria for Adverse Events v4.0 as published by the EORTC (www.eortc.org). Grade I implicates mild AE defined as asymptomatic or mild symptoms; clinical or diagnostic observations only; intervention not indicated. Grade II implicates moderate AE defined as minimal, local or noninvasive intervention indicated; limiting age-appropriate instrumental activities of daily living. Grade III or IV AEs were not observed during this trial. The heading *Type* reports on how the AE was diagnosed; this was either at the injection site (Type I), as a systemic response (Type 2), in the laboratory (Type 3) or otherwise such as pain which cannot be objectified (Type 4). Finally the *duration* of the AE was listed. The description “Ongoing” implicates that the AE was still present at the final follow up visit. Patient number 28 was not mentioned in this table because consent was withdrawn before vaccination 1 and therefore no AE were recorded.

The swelling did not occur until 1.5 weeks after the first vaccination. In all other cases the swelling post vaccination occurred directly or within 1 hour after vaccination. * Because of the pain in the arm after the first vaccination p22 withdrew consent to participate in the trial. The patient stated that this exact pain had also been present before the vaccination but because of the swelling and the fear of worsening of the pre-existing symptoms, the consent was withdrawn

We have previously vaccinated 10 patients with the p53-SLP[®] vaccine but without the administration of IFN- α (6). Clinicopathological parameters of both trial cohorts were similar (table 3). In the current trial all patients developed ongoing swelling at either one or both peptide vaccination sites visible at the final check-up visit (28 weeks after first vaccination), which contrasts with the previous trial in which only one patient showed inflammation at the p53-SLP[®] injection site. In conclusion, addition of IFN- α to the p53-SLP[®] resulted in prolonged and increased inflammation at the vaccination site, suggesting that addition of IFN- α promotes inflammation at p53-expressing sites after injection of p53-SLP[®].

p53-SLP[®] and IFN- α injection elicit both proliferative and IFN- γ producing p53-specific T cells

Using PBMCs isolated from blood samples taken before and after the second vaccination, three complementary T-cell assays (LST, CBA and IFN- γ ELISPOT) were performed to monitor the immunogenicity of p53-SLP[®] combined with IFN- α injection. After the two vaccinations, 4 (p25, p26, p29, p30) out of 9 patients showed vaccine-induced proliferative responses as determined by LST. All 4 patients responded against peptide pool 5, whereas for patients p25 and p26 also responses against peptide pool 3 and in the case of p29, against peptide pool 4 were detected (figure 1A). Based on our cutoff criteria, PBMCs of patient p21 displayed a proliferative response against p53 peptide pools 1 and 6 at baseline that disappeared after vaccination. Patient p27 showed a positive proliferative response against peptide pool 6 after vaccination. However, this response was not induced by vaccination as it may have already been present at baseline, although just below the cut-off. Except for patients p20 and p24, a proliferative response against the recall antigens in the memory response mix was detected both at baseline and after vaccination (data not shown).

Supernatants isolated at day 6 from the cultures of all PBMC samples tested in the LST were used for the analysis of antigen-specific production of cytokines (IFN- γ , TNF- α , IL-2, IL-4, IL-5, and IL-10) by CBA. After vaccination, 6 out of 9 patients (p20, p23, p24, 25, p26, p30) showed detectable induction of IFN- γ (median 55, average 134; range, 26 – 618 pg/mL). IFN- γ production was induced by the vaccine as shown upon stimulation of PBMCs with peptide pool 1 (p26), pool 2 (p24), pool 3 (p20, p24, p25, p26), pool 4 (p24, p30) and/or pool 5 (p20, p23, p25, p26) (figure 1B). One patient (p21) showed IFN- γ production against peptide pool 5 following vaccination, however, also displayed production of this cytokine already prior to the vaccinations. Moreover, the IFN- γ production at baseline was mainly found after stimulation with p53 peptide pools 1 or 6 in patients p21 and p25 (median 251, average 220; range, 23 – 505 pg/mL) (figure 1B). Vaccine-induced production of TNF- α , albeit at very low amounts, was detected in PBMCs of patients p25, p26, and p27 (median 48, average 75; range, 22 – 175 pg/mL). IL-5 production was found in patients p20, p24, p25, p26 (median 33, average 40; range, 22 – 74 pg/mL) and IL-10 in patient p20, p24, p25 and p26 (median 28, average 30; range, 21 – 39 pg/mL) after the vaccinations. No IL-2 was detected, most likely because IL-2

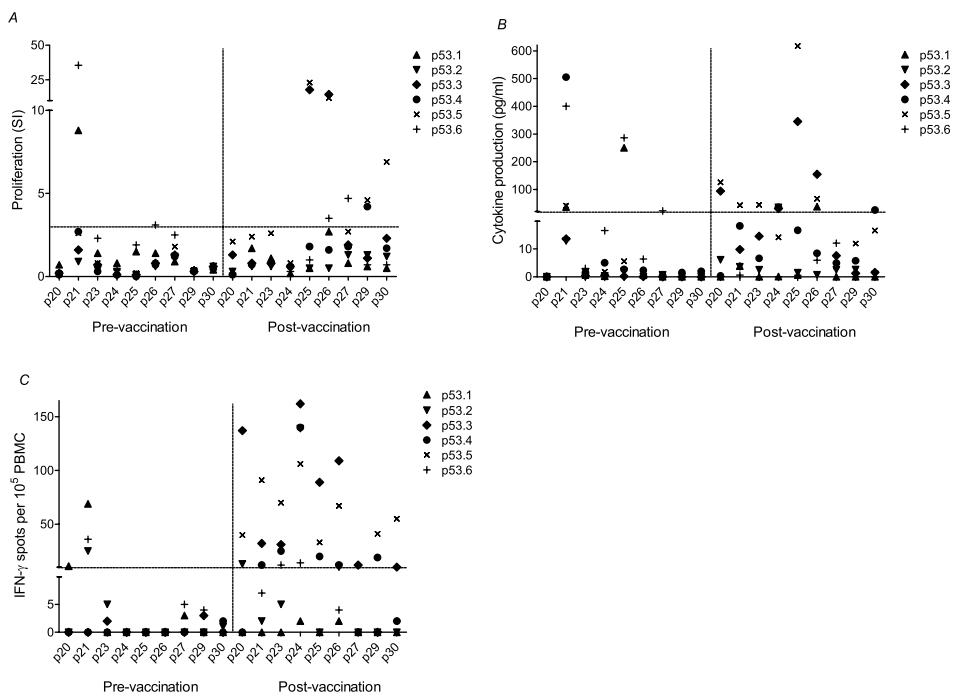


Figure 1

Results of three complementary T-cell assays with either freshly isolated PBMCs: (A) LST and (B) INF- γ in CBA, or cryopreserved PBMCs: (C) INF- γ ELISPOT results are depicted for each individual patient ($n=9$) before vaccination (prevaccination; left) and 3 weeks after vaccination (postvaccination; right); each peptide pool is represented by a symbol. (A) Proliferative responses upon stimulation with the indicated peptide pools of p53 are depicted as stimulation index (SI); a $SI \geq 3$ (*indicated line*) was defined as a positive response. (B) Concentration of INF- γ (pg/ml) as measured by CBA in the supernatants isolated at day 6 from the proliferation assay; production of ≥ 20 pg/ml (*indicated line*) was defined as a positive response. (C) INF- γ ELISPOT results; number of T cells per 10^5 PBMCs specifically producing a spot of the cytokine INF- γ after stimulation with the indicated p53 peptide pools are shown; antigen-specific T-cell frequencies were considered to be positive when specific T-cell frequencies were ≥ 10 of 10^5 PBMCs (*indicated line*)

was consumed by the cells during the 6 days of culture. In none of the cultures, IL-4 could be detected.

The INF- γ ELISPOT assay was used to determine the number of INF- γ producing p53-specific T cells. In all patients, p53-SLP[®] vaccination combined with INF- α injection induced p53-specific T-cell responses (figure 1C). Up to 162 specific spots per 10^5 PBMC against at least one of the vaccine-representing p53 peptide pools were found. Out of 9 patients, 6 patients (p20, p21, p23, p24, p25, p26) displayed an INF- γ -associated T-cell response to at least 3 or 4 peptide pools that represented the vaccine (Figure 1C). In patients p20 and p21, INF- γ -producing T cells were detected in the baseline samples mainly against peptide pools 1 and/or 6 that represented peptides outside the vaccine pool of peptides. These responses were not boosted after vaccination. Five patients displayed an INF- γ -associated

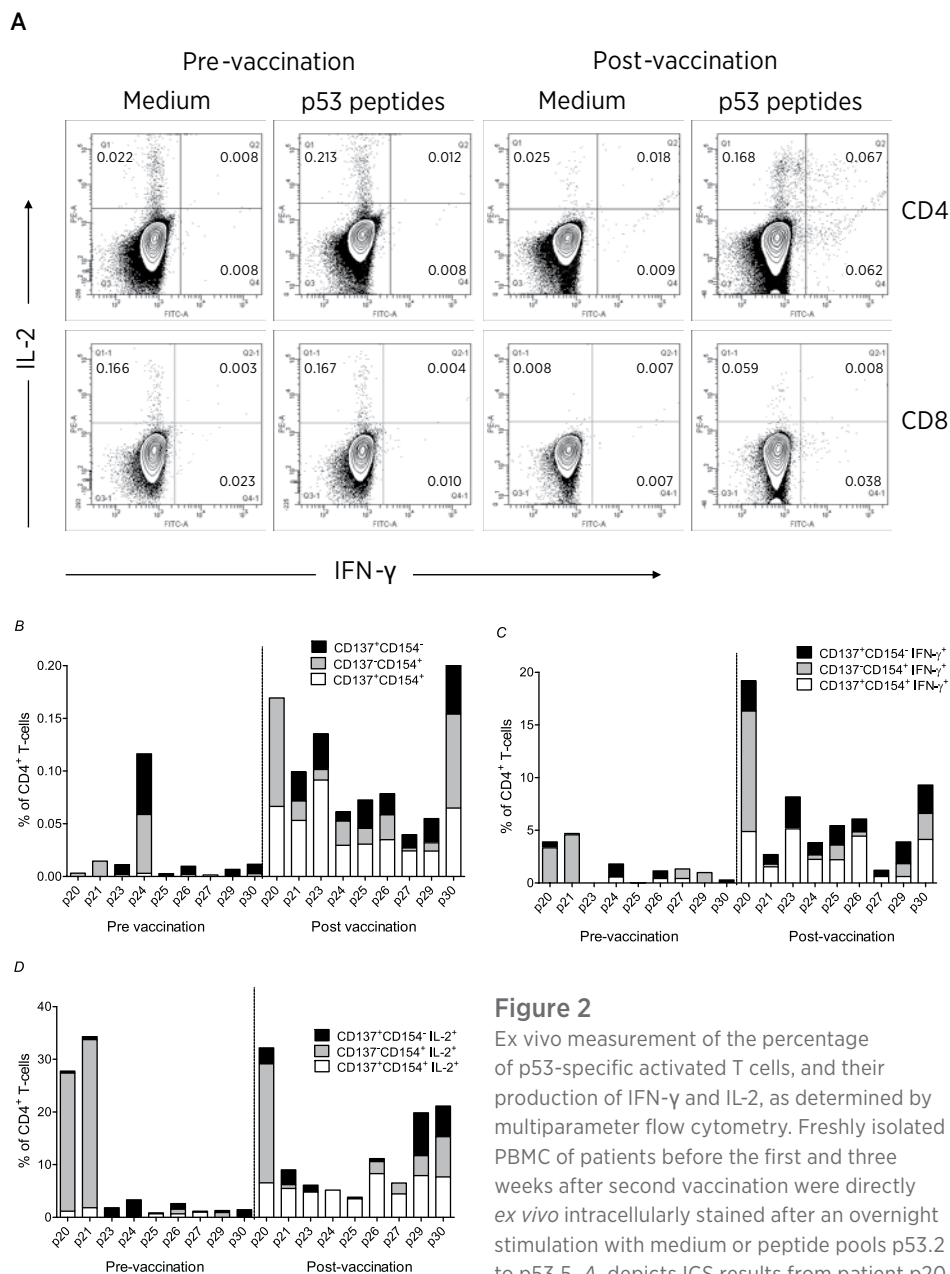


Figure 2

Ex vivo measurement of the percentage of p53-specific activated T cells, and their production of IFN- γ and IL-2, as determined by multiparameter flow cytometry. Freshly isolated PBMC of patients before the first and three weeks after second vaccination were directly ex vivo intracellularly stained after an overnight stimulation with medium or peptide pools p53.2 to p53.5. A, depicts ICS results from patient p20

pre- and post-vaccination. Freshly isolated PBMCs were directly ex vivo stained after incubation in medium or stimulation with p53 peptide pools. Depicted are the IL-2 and/or IFN- γ -producing cells in the CD3⁺CD4⁺ (upper) or CD3⁺CD8⁺ (lower) T-cell population. The numbers in the quadrants indicate the percentage (%) of positive cells within this population. B, displays percentages (%) of p53 specific CD4⁺ T-cells stained positively with the activation markers CD154 and/or CD137; results are given for before (left side) and after vaccination (right side). C and D, display the percentages IFN- γ ⁺ and IL-2⁺ activated CD4⁺ T-cells, respectively split up for the activation markers CD137 and/or CD154, results are given for before (left side) and after vaccination (right side).

T-cell response to the positive control (memory response mix; data not shown). In contrast to patients with p53-negative tumors determined by immunohistochemistry (p27, p29), higher vaccine-induced cytokine levels were found in patients that exhibited p53-positive tumors, as determined by CBA and IFN- γ ELISPOT. In conclusion, the three immune monitoring assays showed that injection of IFN- α in close proximity of the p53-SLP[®] vaccine induced p53-specific IFN- γ -producing T cells in all cases.

Intracellular cytokine staining (ICS) detects p53-specific activated T-cells capable of producing IFN- γ /IL-2

To phenotype and enumerate p53-specific T cells, freshly isolated PBMCs from blood samples drawn before the first and after the second vaccination were directly *ex vivo* stimulated overnight with p53 peptide pools, followed by analysis of the expression of the T-cell markers: CD3, CD4 and CD8; in combination with the T-cell activation markers: CD137 and CD154; and cytokines: IFN- γ and IL-2 by multiparametric flow cytometry (figure 2A). In 8 of the 9 vaccinated patients an increase in the percentage of p53-specific CD4⁺ T cells expressing CD137 and/or CD154 was found after vaccination (figure 2B). In general, the production of the cytokines IFN- γ (figure 2C) and/or IL-2 (figure 2D) in these activated cells was also boosted after vaccination. Notably, the CD4⁺ T cells of patient p24 displayed CD137 and CD154 expression before vaccination, suggesting that this patient already had a pre-existing response to p53, however, only after vaccination these activated T cells produced IFN- γ and IL-2 (figures 2B, 2C, 2D). In 1 out of the 9 tested patients (p20) activated CD8⁺ T cells, which also produced IFN- γ , were detected in the freshly isolated PBMCs obtained after two vaccinations (figure 2A).

T cells cultured from skin biopsies harbor p53-specific reactivity

From 6 out of 9 skin biopsies taken from the second vaccination site sufficient T cells could be cultured to perform multiparametric flow cytometry using the same markers as described above for PBMCs. The CD4⁺ T cells from 5 out of these 6 skin biopsy cultures (p25, p26, p27, p29, p30) displayed elevated expression levels (at least twice the non-stimulated sample) of the activation marker(s) upon stimulation with p53-SLP[®] vaccine-specific peptides (figure 3A); a median frequency of 21% CD137⁺ and/or CD154⁺CD4⁺ T cells could be observed in the p53-peptide stimulated samples versus 2.2% in the non-stimulated T-cell culture control. The vast majority of these activated CD4⁺ T cells produced IFN- γ and/or IL-2 (figures 3B and 3C). Moreover, in biopsies from 3 patients (p25, p26 and p30) both p53-specific IFN- γ producing CD4⁺ and CD8⁺ T cells were found (figure 3D).

Addition of IFN- α to p53-SLP[®] results in increase of IFN- γ producing p53-specific CD4⁺ T cells

The characteristics of the two patient cohorts vaccinated in the current and our previous trial are generally similar (table 3), thereby allowing us to compare the p53-specific immune responses after administration of the two different vaccine

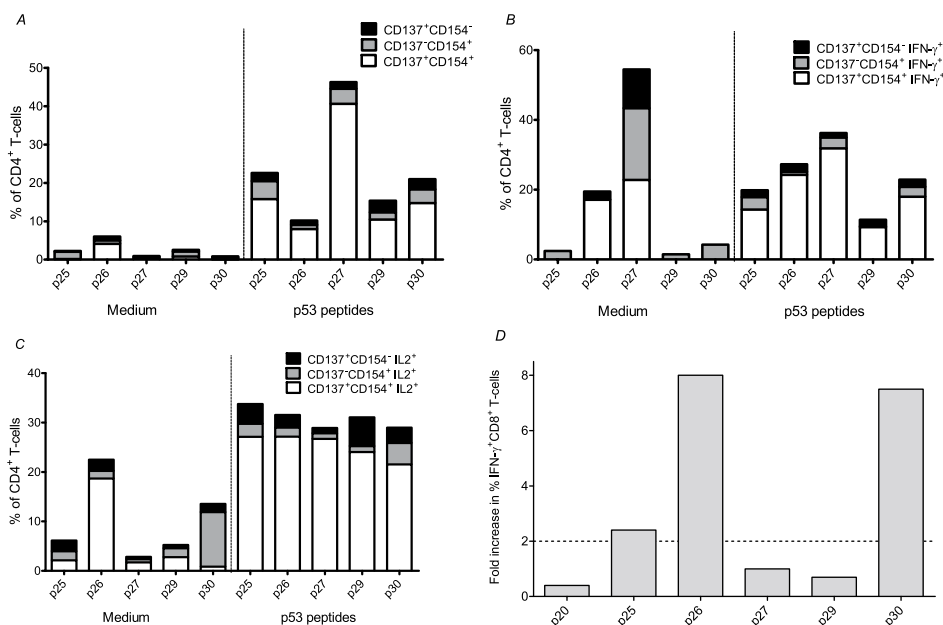


Figure 3

Measurement of the percentage of p53-specific activated T cells cultured from skin biopsies of the p53-SLP[®] injection site, which produce IFN- γ and/or IL-2, as determined by multiparameter flow cytometry. These cultured T cells were incubated in medium or stimulated with peptide pools p53.2 to p53.5. *A*, displays percentages (%) of CD4⁺ T cells stained positively with the activation markers CD154 and/or CD137. *B* and *C*, respectively display the percentages IFN- γ ⁺ and IL-2⁺ activated CD4⁺ T-cells split up for the activation markers CD137 and/or CD154. *D*, depicts the ratio of IFN- γ ⁺CD8⁺ T-cells of p53 peptides versus medium stimulated T cells. The line indicated a 2-fold increase; > 2-fold increase is defined as a positive response.

modalities in terms of their immunogenicity, with the limitation that the two vaccine modalities were not directly compared in the same trial.

To enable comparison of the results with those from the first trial, available cryopreserved PBMCs from patients in the first clinical trial (i.e. p01, p02, p03, p04, p07, p10, p11), vaccinated with p53-SLP[®] only, and cryopreserved PBMC samples from the patients in the current trial were thawed and subsequently head-to-head tested in a direct ex-vivo ICS assay, optimized for detecting both antigen-specific CD4⁺ and CD8⁺ T-cell responses in one single cryopreserved PBMC sample using long overlapping peptides as antigens (25) (figures 4A-D). In PBMCs from patients receiving p53-SLP[®] in combination with IFN- α not only significantly more CD154⁺CD4⁺ were found ($p=0.002$), but also a significantly higher frequency of these activated CD4⁺ T-cells produced IFN- γ ($p=0.008$), when compared to the activated CD4⁺ T cells isolated from patients that received the p53-SLP[®] vaccine only. Importantly, in 4 out of 9 patients (p20, p25, p27 and p29), who received p53-SLP[®] and IFN- α , also low numbers of IFN- γ -producing CD8⁺ T-cells were found, while patients vaccinated with p53-SLP[®] only, showed no p53-specific CD8⁺ T-cell reactivity (Figure 4D). Of note, cryopreserved PBMCs, stored in the vapour phase

Table 3Comparison of patient characteristics p53 vaccination study with and without IFN- α

Characteristic	p53-SLP® (n=10)	P53-SLP® + IFN- α (n=9)
Sex (%male)	8 (80%)	6 (68%)
Age (average, years)	61	58
TNM (%)		
1/2	0 (0%)	1 (11.1%)
3	5(50%)	2 (22.2%)
4	5(50%)	6 (66.7%)
Location primary (%)		
Cecum	1(10%)	1(11.1%)
Ascending colon	0(0%)	2(22.2%)
Transverse colon	1(10%)	0(0%)
Sigmoid colon	4(40%)	1(11.1%)
Rectum	4(40%)	5(55.6%)
Location 1 st metastasis (%)		
Liver	8(80%)	8(88.9%)
Lung	1(10%)	1(11.1%)
Liver+Lung	1(10%)	0(0%)
P53 Status (IHC)*		
1	4(40%)	2(25%)
2	2(20%)	3(37.5%)
3	4(40%)	3(37.5%)
CEA (screening, average)	3,7	2.2

Abbreviations: TNM: Tumor Node Metastasis Stage at time of diagnosis primary tumor. p53 status IHC. 1: expression of p53 in <25% of the tumor cells; 2: expression of p53 in $\geq 25\%$ but <75% of the tumor cells; 3: expression of p53 in $\geq 75\%$ of the tumor cells. * in patients vaccinated with p53-SLP® and IFN- α there was insufficient tissue present for IHC in 3 patients. CEA: Carcinoembryonic Antigen.

of liquid nitrogen, reacted similarly over a period of at least 4 years indicating that the influence of cryopreservation time is unlikely (data not shown).

ELISPOT plates from the previous trial were reanalyzed using the same ELISPOT reader conditions as the current trial to obtain a fair comparison. Addition of IFN- α to the p53-SLP® clearly results in a broader response per vaccinated patient (figure 5A). Patients that were injected with both p53-SLP® and IFN- α showed a significantly higher median frequency of IFN- γ producing T cells after vaccination ($p=0.018$) compared to patients that received the p53-SLP® only vaccine (figure 5B). These data recapitulate the results obtained in the direct ex-vivo ICS assay conducted on cryopreserved PBMCs.

Comparison of the IgG responses to p53 in serum of p53-SLP® vaccinated patients with and without IFN- α

In order to analyze whether vaccination also resulted in the induction of a p53 peptide-specific antibody response we developed a p53 peptide-specific ELISA and subsequently analyzed the sera of the patients obtained prior to the first and

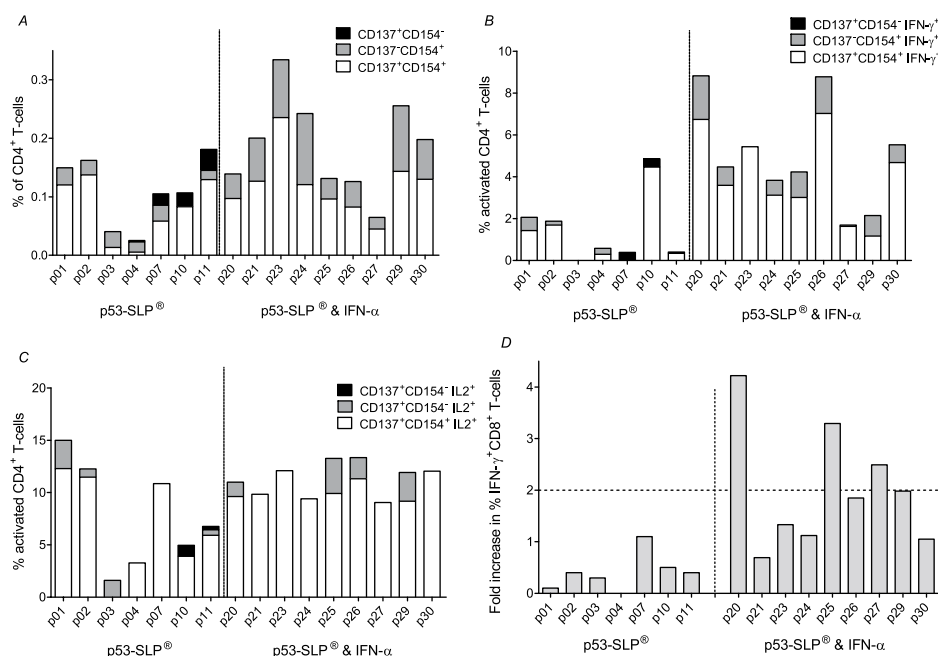


Figure 4

Results from patients injected with p53-SLP[®] and IFN- α compared to the results from colorectal cancer patients injected with p53-SLP[®] only. *A*, The bar plot shows a graphical comparison of the percentages of activated CD4⁺ T-cells (CD137⁺CD154⁺ in black, CD137⁺CD154⁺ in gray and CD137⁺CD154⁺ in white) between the two vaccination studies. *B* and *C*, display the percentages IFN- γ ⁺ and IL2⁺ activated CD4⁺ T-cells respectively split up for the activation markers CD137 and/or CD154. *D*, depicts the ratio of IFN- γ ⁺CD8⁺ T-cells of p53 peptides versus medium stimulated PBMC. The line indicated a 2-fold increase; > 2-fold increase is defined as a positive response.

after the second vaccination. These analyses were performed simultaneously on the sera obtained from patients participating in the current trial and our previous vaccination study. In the current trial p53-specific IgG antibody responses were detected in 7 out of the 8 patients of whom both serum samples (pre- and post-vaccination) were available. In one patient (p29) no antibody response to p53 was detected. On average, the number of p53 peptides to which IgG antibodies were detected in these 8 patients was 2.3 (range 0-5), with an obvious peak in the recognition of those peptides that were present in the vaccine (peptides 9-15; figure 6). In sera of 3 out of the 9 tested patients from our previous colorectal cancer trial p53-specific IgG responses were detected. Here on average the number of peptides recognized was 0.4 (range 0-2). Patients in the current trial recognized significantly more peptides than those from the first trial ($p=0.02$). The results of the ELISA therefore indicate a broader p53-specific IgG response by the addition of IFN- α to the p53-SLP[®] vaccine.

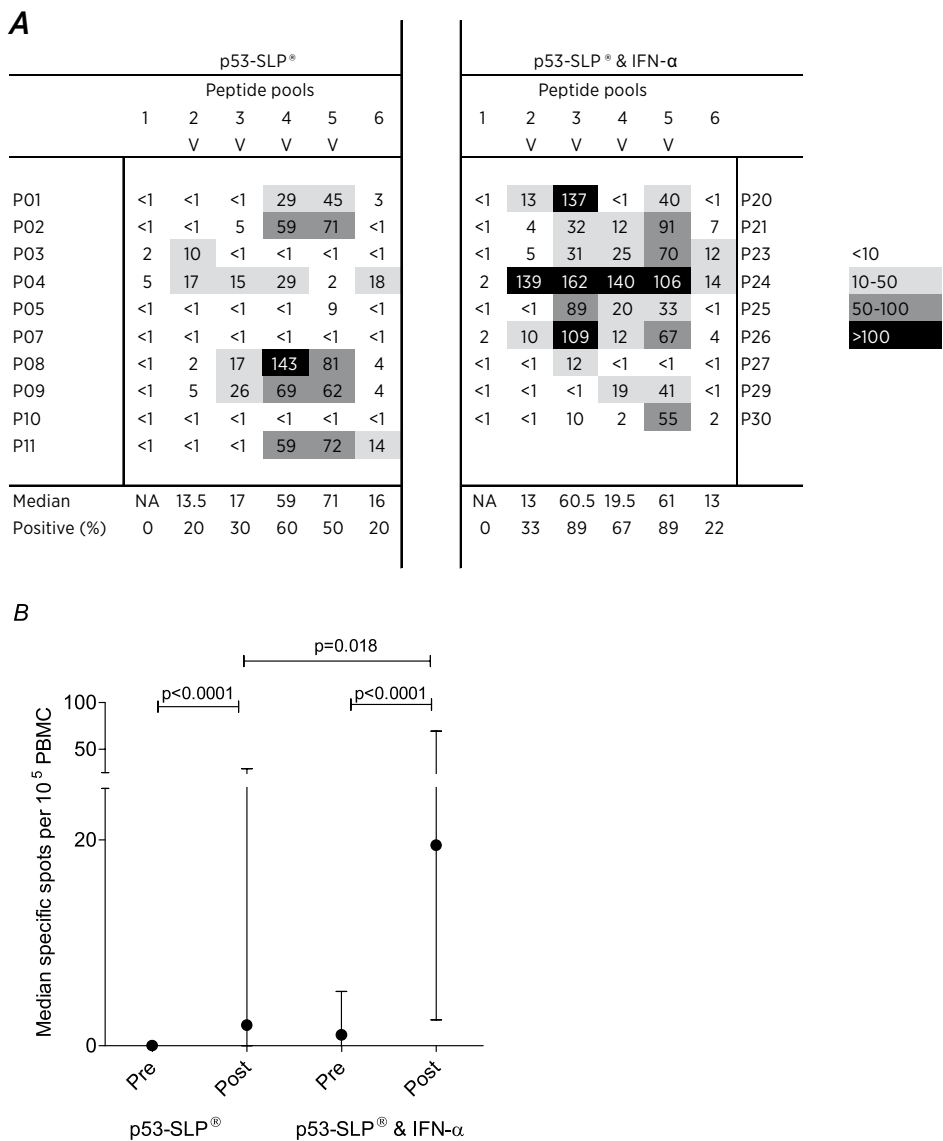


Figure 5

The heat map of (A) reflects the IFN- γ ELISPOT results from both trials. The number of positive spots per 10^5 PBMC is given for every patient. Every value is colored in relation to the number of positive spots per 10^5 PBMC. White corresponds with a count of < 10 positive spots 10^5 PBMC, light grey ≥ 10 - < 50, dark grey ≥ 50 - < 100 and black ≥ 100 spots per 10^5 PBMC. On the bottom of the heat map the median and the percentage positive responses is given per peptide pool. Peptide pools represented by the vaccine are indicated by a 'V'. (results of patients vaccinated with only p53-SLP* (left side) and vaccinated with p53-SLP* and IFN- α (right side). (B) The median of all specific spots as determined IFN- γ ELISPOT in the two trials before and three weeks after the second vaccination are compared by reanalyzing the ELISPOT plates of the first trial (p53-SLP* only) with the same settings of the reader as the current trial (p53-SLP* and IFN- α). Not only the number of specific spots in both cohorts was significantly higher after vaccination compared to pre-vaccination, this increase in specific spots was significantly better in the patients that also received the IFN- α injections besides the regular vaccine.

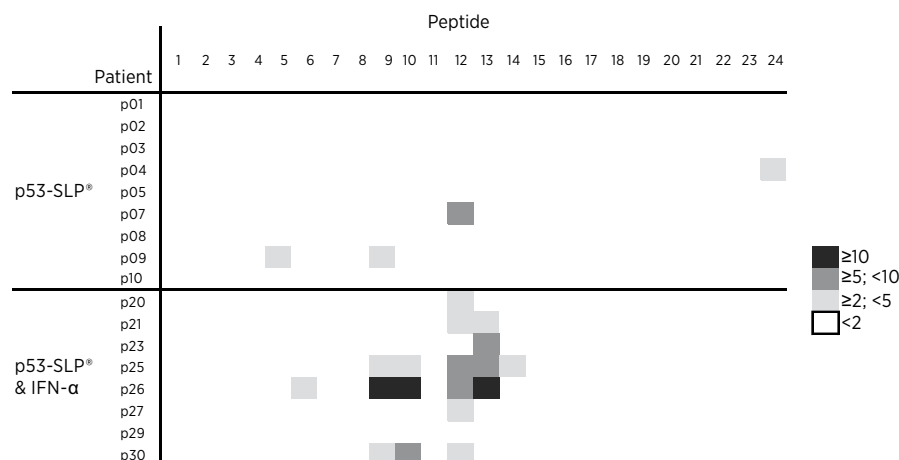


Figure 6

The grid table in this figure represents the number of peptides that were recognized by the individual patients based on their specific IgG antibody responses. The peptides are listed on the x-axis, the individual patients on the y-axis. Peptides are numbered and indicated by the first and last amino acids used of the p53 protein. Patients 1-10 represent the study cohort of the first trial who received solely p53-SLP[®] injections, patients 20-30 represent the study cohort of the second trial in which patients received both p53-SLP[®] and IFN- α injections. Negative responses are white (< 2); positive responses are displayed in light grey: ≥ 2 and < 5, dark grey ≥ 5 and < 10-fold and black ≥ 10 -fold increase of p53-specific IgG response after vaccination. There is an obvious peak, mainly present in the second cohort, in the recognition of peptides that were actually covered by the vaccine.

DISCUSSION

Results from previous studies suggest that colorectal cancer vaccines should aim at inducing strong type 1-associated immunity to obtain a clinical response (11,33-35). Although in patients, vaccination with the p53-SLP[®] resulted in the induction of p53-specific CD4⁺ T-cell immunity, the production of Th1-associated cytokines such as IFN- γ and IL-2 was probably too low to become truly effective (6). Combining vaccines with immune modulating adjuvants should allow polarization of the vaccine-induced immune response. Here we show that the clinical grade p53-SLP[®] vaccine combined with IFN- α induced p53-specific Type 1-polarized CD4⁺ and CD8⁺ T-cell responses in all and 6 of 9 colorectal cancer patients, respectively. We have previously shown that the p53-SLP[®] vaccine was safe (6,7). The addition of IFN- α in the current trial also resulted in no serious AEs. However, in contrast to vaccination with p53-SLP[®] only (6), this time all vaccinated patients showed long lasting local swelling and inflammation of at least one of the peptide but not the IFN- α injection sites. As the groups of vaccinated patients were highly comparable (table 3), this suggests that addition of IFN- α potentiates inflammation at the vaccination sites where p53 antigen is present, thereby improving the antigen presentation conditions and subsequently the priming of T cells.

The vaccine-induced p53-specific antibody and cellular response of patients vaccinated with the combination p53-SLP® and IFN- α were compared with those of patients vaccinated with p53-SLP® only. They were analyzed in a head-to-head comparison of cryopreserved PBMC samples by ICS and serum samples in the peptide ELISA assay. Although, these materials were obtained in two independent trials, they were similarly isolated from successfully treated colorectal cancer patients with highly comparable disease state and preserved under the same conditions. Within these limitations, our results indicate that addition of IFN- α to p53-SLP® induces an immune response against a broader range of peptide pools and also a higher frequency of vaccine-specific activated IFN- γ producing T cells. Addition of IFN- α to p53-SLP® also increased the amount of p53-specific IgG antibodies, indicating the underlying improved Th cell induction.

In the current trial, one of the peptides was excluded from the original p53-SLP® vaccine composition, due to low yield of purified material of this particular long peptide (6). The peptide not included was the last 13 amino acid overlapping peptide from the C-terminal section of the p53 sequence used in the previous vaccination trial (6). Our data comparing p53-specific T-cell responsiveness was not focused on the measurement of responses to individual peptides, therefore, it is difficult to estimate how the exclusion of this specific peptide altered the immunogenicity of the vaccine. However, in the current trial the responsiveness after vaccination was significantly increased compared to the previous trial, despite the lack of this one peptide.

In the literature, it has been suggested that the p53-specific CD8⁺ T-cell repertoire is severely restricted due to self-tolerance (36,37). Consequently, p53-specific vaccination will result mainly in the induction of p53-specific high affinity CD4⁺ T-cells and low affinity CD8⁺ T cells. Our results indicated that addition of IFN- α might have increased the number of p53-specific CD8⁺ T cells as we were able to detect them in 6 out of 9 patients from the present trial and in none of the patients from the previous trial. The 6 patients with p53-specific CD8⁺ T cells included the following 4 patients: one patient (p20) who showed a response when PBMC were freshly tested and the others (p25, p26 and p30) displayed p53-specific CD8⁺ T cells in the cells cultured from the biopsy of the vaccine site. Most CD8⁺ T-cells responses were found in cryopreserved PBMCs (p20, p25, p27 and p29). The reason why we were better able to detect p53-specific CD8⁺ T cells in the cryopreserved samples lies in the fact that the ICS assay used for analyzing thawed PBMC is optimized to detect antigen-specific CD8⁺ T cells by using 10-fold higher concentrations of the long peptides as antigens. It also differs from the assay used to analyze the fresh PBMC samples by the addition of TLR3 agonist poly I:C to activate the peptide-loaded antigen presenting cells (25).

Together, we have found that combining p53-SLP® with IFN- α injection results in enhanced inflammation, p53-specific type 1-polarized CD4⁺ and CD8⁺ T-cell responses. We have not studied the effect on DC activity, therefore we can only speculate on the exact function of IFN- α . However, from literature it is clear that IFN- α improves antigen cross-presentation (38) and enhances survival of activated

T cells (39). A recent study also found a reduction in regulatory T cells following high-dose IFN- α (40).

We can conclude that the addition of IFN- α clearly induces both a qualitatively and quantitatively better p53-specific T-cell response compared to p53-SLP® vaccination alone. These data provide support to the notion of combining cancer vaccines with immune modulating agents such as IFN- α to augment and polarize the vaccine-induced immune response. However, the minimal requirements of a vaccine-induced immune response in order to obtain a clinical response are undefined. Therefore it is tempting to perform an efficacy study with p53-SLP® combined with IFN- α to determine whether the strength and quality of the response are good enough to prevent recurrence or metastasis in stage II and stage III colorectal cancer patients, who have not yet developed any kind of distant metastasis at the time of vaccination.

ACKNOWLEDGEMENTS

The peptides used in this study were kindly provided by ISA Pharmaceuticals B.V., Leiden, the Netherlands, and this study was supported by a personal grant from The Netherlands Organisation for Health Research and Development (to F.S)

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CHAPTER 8

FoxP3- and CD8-positive infiltrating immune cells together determine clinical outcome in colorectal cancer

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Cancer Microenvironment. 2011: 6 31-39

ABSTRACT

Immune cells are known to affect clinical outcome in colorectal cancer. Subsets of immune cells can both support and inhibit immunological interaction with tumor cells. We examined the clinical impact of T cells that are supposed to be responsible for the down regulation of a T cell response: regulatory T cells or Tregs. The study population (n=76) consisted of a random population of colorectal cancer patients who did not receive any (neo-) adjuvant therapy, with a median follow-up time of 7.3 years (range 0.1-23.1 years). Expression of FoxP3 was used as an immunohistochemical marker to identify Tregs. We considered FoxP3+ cells present in tumor stroma and tumor epithelium separately, and related results to clinical outcome and to data on CD8+ immune cell infiltration that we previously obtained in the same patient cohort. All samples showed presence of Foxp3+ cells and in the majority of the patients (85.5%) these cells were also present in the tumor epithelial compartment. A relative high level of Foxp3+ cells in the tumor epithelium was significantly related to down regulation of HLA Class I expression (p -value 0.03). There was a trend, but no significant relation, towards a longer overall survival (p -value; 0.084) and disease-free survival (p -value; 0.073) when high levels of Foxp3+ cells were present in the tumor epithelium. More importantly, the ratio of CD8+/Foxp3+ cells did show a significant correlation with distant-recurrence-free survival. This was the case for both Foxp3+ cells specifically located in the tumor epithelium (p -value 0.024) as well as in the stroma compartment (p -value 0.018). Unfortunately due to the small sample size the ratios did not retain their statistical significance in multivariate analysis. These results provide further evidence that local interactions in the cancer microenvironment between tumor cells and immune cells are not only determined by tumor cell-related factors like HLA expression, but also by interactions among immune cells.

INTRODUCTION

The process of tumor development in colorectal cancer (CRC) is one of the most studied and best characterized models of tumorigenesis in the world today. At least six features can be identified that hallmark tumor growth and development. (1;2) Three of which are related to the immune system. Tumor cells may acquire the ability to thrive in a inflamed micro-environment, to evade immune recognition and, to suppress immune reactivity. (3)The adaptive immune system and its key players, especially cytotoxic T Lymphocytes (CTL), play an important role in controlling tumor growth and metastasis. Naito *et al.* were the first to demonstrate that infiltrating CTL are a prognostic factor in CRC. (4) Due to their genetic instability, tumor cells may arise with a phenotype that makes them less sensitive to immune surveillance, thus escaping CTL recognition. This phenotype includes down regulation or complete loss of one or more HLA class I alleles. This enables tumor cells to minimize their presentation of tumor-associated antigens (TAA) and, therefore, diminish their chance of recognition and subsequently destruction by CTL. (5-8) Another mechanism to evade immunosurveillance is to suppress immune reactivity by attraction of immunosuppressive regulatory T cells (Tregs) into the tumor micro-environment. Both, down regulation of HLA class I, and presence of Tregs, have been shown to be of clinical relevance in various types of solid tumors. (9-12) CRC-related studies that describe HLA class I expression in relation to clinical prognosis have, however, rendered contrasting results. Some studies were not able to determine any significant relation between HLA class I expression and survival, while others did. (13-16) When a significant relation could be established, a total lack of HLA class I expression always resulted in more favorable prognosis. This is probably due to the fact that HLA Class I negative metastatic cells in the circulation will be targets for destruction by Natural Killer (NK) cells that are abundantly present in the circulation but not in solid tumors. (17;18) Partial expression, however, was related to either a better or worse prognosis compared to high expression (15;16) Similar, there are a variety of studies describing the presence of Tregs in tumor tissue in relation to clinical outcome. In case of ovarian cancer, three studies were able to establish that high Treg infiltration was associated with a poor prognosis. (10;19;20) In colorectal cancer, however, 4 studies describing the presence of Tregs in cancer tissues have rendered contrasting results. Loddenkemper *et al.* performed an analysis on 40 colorectal tumor samples and could not establish a significant relation between the presence of Foxp3⁺ Tregs or the ratio CD8⁺/Tregs with outcome.(21) In contrary, a recently published study by Suzuki *et al.*, a predictive value of the intra-tumoral ratio of CD8⁺ T cells and Foxp3⁺ Tregs could be established.(22) A third study by Salama *et al.* using a Tissue Micro Array of 967 stage II and III CRC patients was able to significantly correlate the presence of Tregs to clinical outcome parameters. (23) High Foxp3⁺ Treg density in the normal mucosa of patients was associated with a worse prognosis, while a high density in the tumor tissue was associated with improved survival. Finally, a study of Deng *et al.* was able to significantly correlate the accumulation of Foxp3⁺ Tregs in draining

lymph nodes to disease.(24) The latter four publications as well as the studies on the significance of HLA Class I expression in colorectal cancer all have in common that they were performed on mixed study populations containing both adjuvant and non-adjuvant treated, metastatic and non-metastatic patients and patients of which tumor microsatellite status was often not determined. Besides, the presence of Foxp3+ Tregs was expressed in several ways; either as their presence in the tumor epithelium, in the tumor stroma or as a ratio with CD8+ cells. All these variations in study methods make it difficult to recapitulate the results of these studies into one overall conclusion. Therefore, the importance of HLA class I expression and Foxp3+ Treg infiltration in relation to the presence of CD8+ CTLs still remains to be determined in CRC patients. The purpose of this study was to analyze the presence of Foxp3+ Tregs and relate the data to the data on infiltrating immune cells and HLA expression that we have previously obtained in a random cohort of patients, without adjuvant treatment.(7) The data on Foxp3+ Tregs were also correlated with clinico-pathologic variables such as TNM (Tumor Node Metastasis) classification, tumor differentiation grade and the tumors microsatellite stability status (MSS/MSI), as well as with outcome parameters such as overall survival (OS) and disease free survival (DFS).

PATIENTS AND METHODS

Patients

Between January 1980 and December 1992, 266 consecutive patients underwent curative resection with post-operative follow up for a colorectal adenocarcinoma at the Leiden University Medical Center. Within this cohort, previously described by Tollenaar *et al.* and Menon *et al.*, 76 patients were identified who did not receive any (neo-) adjuvant therapy and of whom data was available on CD8+ lymphocyte infiltration, tumor microsatellite stability status and HLA Class I expression. (7;15;25) CD8+ infiltration was scored as the number of CD8+ leukocytes per tumor area (leukocytes/mm² tumor epithelium). The average leukocyte infiltration of 25 fields per tumor section was calculated and defined as the intraepithelial leukocyte infiltration. In addition, leukocytes in the tumor stroma and at the tumor margin were assessed semi quantitatively into 'none-poor' or 'moderate - marked' leukocyte infiltration. The tumor microsatellite status was determined with MLH1-based immunohistochemistry. MLH1 expression was categorized as absent if the majority of the tumor cells lacked nuclear MLH1 expression; this suggests a microsatellite instable (MSI) tumor phenotype. If nuclear MLH1 expression was present in the majority of the tumor cells samples were indicated to be microsatellite stable (MSS).

HLA Class I expression was determined previously by immunohistochemistry using the monoclonal antibodies HCA2 and HC10 according to the defined standard method of the International HLA and Immunogenetics Workshop (IHIW) in which HCA2 and HC10 staining are scored in 5 categories. (0-5% positivity,

5-25% positivity, 25-50% positivity, 50-75% positivity and 75-100% positivity). In 66 out of the 76 (87%) tumors, both HCA2 and HC10 staining could be quantified and, therefore, were available for evaluation of total HLA Class I expression. Three groups were identified according to a standardized method and defined as (1) HLA class I loss (staining of both HCA2 and HC10 was less than 5% of the tumor cells; (2) HLA class I reduced expression (staining of either HCA2 or HC10 was less than 50% of the tumor cells); and (3) HLA class I expression (staining of both HCA2 and HC10 was more than 50% of the tumor cells).(8)

Tumors were staged according to the TNM criteria.(26) Known tumor characteristics included differentiation grade, Jass lymphocyte infiltration score, DNA micro-satellite status, and presence of mucinous characteristics. Median follow-up was 7.3 years (range 0.1 -23.1 years). Patient and tumor- characteristics are shown in table 1.

Immunohistochemistry

A Foxp3 / laminin immunohistochemical double staining of tumor sections was performed to evaluate the presence of Foxp3+ regulatory T cells in both the epithelial and stromal compartment of the primary tumors. FFPE (formalin-fixed paraffin-embedded) tissue sections of 5 µm thickness were prepared on amino-propylethoxysilane (APES)-coated slides and dried at 37°C. Tissue sections were deparaffinized in xylene, submerged in 0.3% hydrogen-peroxidase methanol to block endogenous peroxidase, and rehydrated in a graded ethanol series. For antigen-retrieval, tissues were soaked in 0.01 M citrate buffer (pH 6) at 100°C for 10 minutes. After rinsing in PBS, tissues were incubated overnight with anti-FOXP3 (1:300, ABCAM AB20034). On the second day, Biotin-labeled Rabbit-anti-Mouse secondary antibody (30 minutes, 1:200, DAKO) followed by Streptavidin-Biotin-complex (30 minutes, 1:100, DAKO) was used for signal amplification. After 5 minutes of soaking in a 0.05M Tris-HCl solution, tissues were developed for 10 minutes with 3,3-di-amino-benzidine tetrahydrochloride (DAB) for visualization. Next, tissues were transferred to a 0.01% trypsin solution and soaked at 37°C for 10 minutes. Rabbit polyclonal anti-laminin (1:50, Sigma L9393) was applied to the sections for overnight incubation. Subsequently, tissues were incubated with Biotin-labeled Swine-anti-Rabbit (1:400, DAKO) for 30 minutes, followed by Streptavidin-Biotin-complex (1:100, DAKO) for 30 minutes. A blue signal was achieved by developing the tissue sections for 12 minutes in a 4-chloro-1-naphtol solution. Methyl green was used for nuclear counterstaining. Finally, all tissue sections were dehydrated and mounted in Kaiser's glycerol. FFPE sections of tonsil and normal colon tissues were included as positive controls for the Foxp3 and laminin staining and as negative controls by replacing incubations with either the primary antibody or secondary antibody by mock incubations.

Table 1
Clinico-pathological characteristics of the 76 patients median follow-up 7.3 yrs (range 0.1-23.1yrs)

Characteristic	N	(%)
Sex		
male	44	57.9
female	32	42.1
Age (yrs)		
median	69.8	
range	31 - 85	
Tumor location		
colon left-sided	29	38.2
colon right-sided	29	38.2
rectum	19	23.7
TNM Stage		
I	31	40.8
II	12	15.8
III	33	43.4
Differentiation grade		
poor	42	56.8
moderate	18	24.3
well	14	18.9
unknown	2	-
Mucinous Characteristics		
yes	19	27.5
no	50	72.5
unknown	7	-
Jass Lymphocyte Infiltration		
absent / scarce	23	33.3
moderate / marked	46	66.7
unknown	7	-
Microsatellite stability		
MSS	63	82.9
MSI	13	17.1
HLA status		
High expression	43	65.2
Reduced expression	18	27.3
Loss of expression	5	7.6
unknown	10	-

Abbreviations. Yrs: years. TNM: Tissue Node Metastasis. MSS: Micro Satellite Stable. MSI: Micro Satellite Instable. HLA: Human Leukocyte Antigen.
This table shows the clinico-pathological characteristics of all of the 79 primary colorectal cancer patients included in this study.

Evaluation of the Immunohistochemistry

The number of Foxp3+ cells in primary colorectal tumor tissues was microscopically evaluated by two independent observers (ECMZ and AQvH). Microscopic evaluation was done without previous knowledge of patient characteristics or disease outcome. The laminin-staining served as demarcation to distinguish the epithelial from stromal tumor compartments and other tissue. For counting, an ocular grid was used at 100x magnification. First the total amount of Foxp3+ cells (tot-Foxp3+) was counted in 5 randomly chosen, separate grid fields (surface-area of 3mm²) in each tumor. This approach enabled to determine the number of positive cells per mm² tumor tissue. The tot-Foxp3+ count was further subdivided according to location of the Foxp3+ cells: Foxp3+ cells located actually in the tumor epithelium, which means there was direct contact with the tumor cells and the Foxp3+ cells, were counted as tumor-Foxp3+ cells (t-Foxp3+), and Foxp3+ cells located in the tumor stroma as stromal-Foxp3+ cells (s-Foxp3+). Inter-observer conformity was calculated using the intraclass correlation test. Intraclass correlation coefficients for single measures were 0.93, 0.82 and 0.93 ($p < 0.001$) for the tot-Foxp3+, the t-Foxp3+ and the s-Foxp3+ cell counts respectively. Observer counts were averaged and used to calculate the final t-Foxp3+ and s-Foxp3+ counts and the t-Foxp3+/tot-Foxp3+ ratio for each tumor sample. Previously determined number of CD8+ cells present in the tumor epithelium (t-CD8+) were used to calculate the t-CD8+/ t- or s-Foxp3+.(7)

Statistical Analysis

Statistical analyses were performed with SPSS software version 16.0 (SPSS Inc, Chicago, IL). To test whether the mean levels of Foxp3+ cells differed between the tumor and the stroma compartments the Wilcoxon Signed ranks test was applied. The Chi-squared test was used to evaluate associations between the presence of t-Foxp3+ cells, s-Foxp3+ cells and the t-Foxp3+/tot-Foxp3+ ratio and various clinico-pathologic parameters, and HLA Class I expression and the presence of CD8+ cells. For the survival analysis, date of surgery was used as the entry date for analysis. The Kaplan-Meier method was used for overall survival (OS), disease-free survival (DFS), distant-recurrence-free survival (DRFS) and local-recurrence-free Survival (LRFS) and the log-rank test for comparison of the survival curves. OS, DFS, DRFS and LRFS were defined as follows; date of any event of interest minus entry date. Hazard ratios were calculated the Cox proportional hazards analysis for uni- and multivariable analysis. Only parameters that were of statistical significance in the univariate analysis were analyzed in the multivariate model. Significance of hazard ratios was tested with the Wald's test. All statistical tests were two-tailed with a 0.05 significance level.

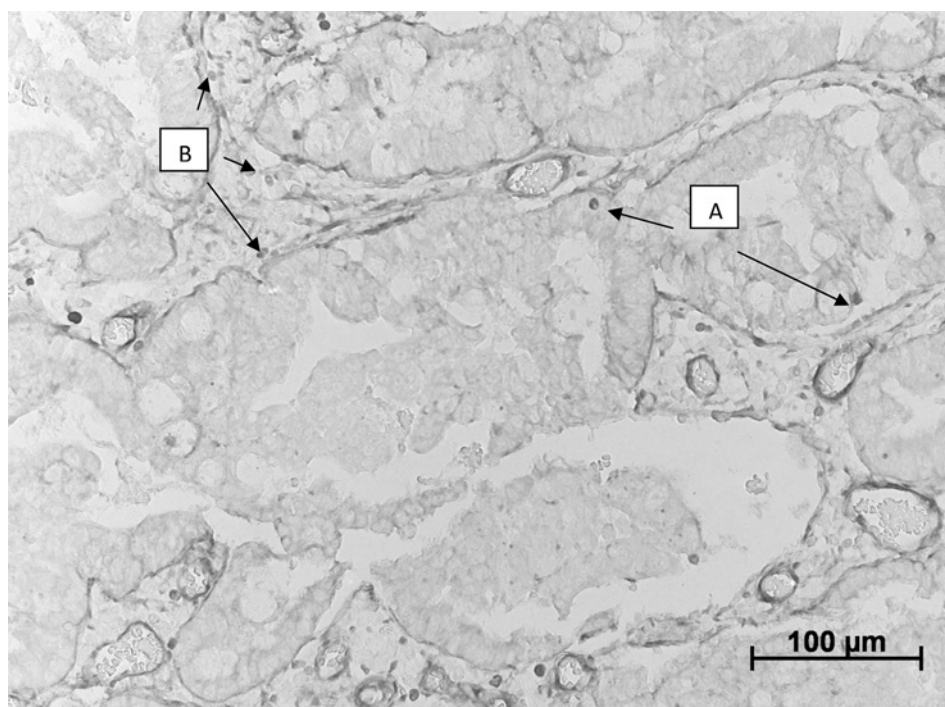


Figure 1

A representative image of the Foxp3 and laminin double staining. In which the laminin clearly demarcates the tumor stroma from the tumor epithelium. In this image typical examples of epithelial Foxp3+ cells (A) and Foxp3+ cells in the tumor stroma can be identified (B).

RESULTS

Immunohistochemistry

The laminin IHC double staining demarcated the tumor epithelium from the stromal compartment within the tumors. (figure 1) Therefore, the exact location and number of the Foxp3+ cells within the tumor tissue could be determined. For all 76 cases, Foxp3 infiltration data were obtained. Foxp3+ cells were found to be present in the stromal compartment of all tumors. In the majority of the patients (85.5%) Foxp3+ cells were also present in the tumor epithelium. The mean number of Foxp3+ cells in the tumor epithelium (t-Foxp3+) was 0,25/mm² with a range of 0-1.73, and in the tumor stroma (s-Foxp3+) 5.84/mm², ranging from 0,43-20,87. This was a significant difference (p -value <0.000). Because the counts of both locations of FoxP3+ cells were skewed to the right of the range, the median was used for further analysis as a cut-off point for the presence of Foxp3+ cells. The median was 0.133/mm² for t-Foxp3 and 4.818/mm² for s-Foxp3 respectively.

The numbers of CD8+ cells were previously determined in the same patient cohort. As described in the study by Menon *et al.*, the cut-off used for the number of CD8+ cells in the tumor epithelium (t-CD8+), was at the 75 percentile (65 cells/mm²)

(7). The presence of CD8+ cells at the tumor margin (m-CD8+) or tumor stroma (s-CD8+) was scored qualitatively as either no or poor infiltration, moderate, and marked infiltration. The scores in the entire study population did not statistically significantly differ from those of the patients used in this subset analysis.

Association of presence and location of Foxp3+ cells with clinico-pathological parameters, HLA class I expression, and presence of CD8+ cells.

Neither the presence of t-Foxp3+ or s-Foxp3+ showed a significant correlation to any of the standard clinico-pathological parameters (table 2). Their presence was also not related to HLA class I expression status of the tumor. The ratio of t-Foxp3+/tot-Foxp3+ did show a statistically significant relationship with HLA class I expression. When HLA class I expression was low, no expression or reduced expression within the tumor, the ratio of t-Foxp3/tot-Foxp3 was higher at a *p*-value of 0.03 (table 2). Patients with reduced HLA class I expression were therefore more likely to have relatively higher numbers of Foxp3+ cells in the tumor epithelium compared to the number of s-Foxp3.

In concordance with the data previously shown by Menon *et al.*, the number of t-CD8+ cells significantly correlated with tumor stage and was inversely correlated with HLA status. (7)

The presence of t-Foxp3+ cells or the ratio of t-Foxp3+/tot-Foxp3+ cells did not show any statistical significant relationship with the presence of CD8+ cells at any location in the tumor. In contrast, the presence of s-Foxp3+ cells did show a significant relation to the presence of CD8+ cells at the tumor margin. When more CD8+ cells were present in the tumor margin the number of Foxp3+ cells in the stroma also increased statistically significantly (*p*-value: 0.043, table 3).

Association of the t-CD8+/Foxp3+ ratio with clinico-pathological parameters and HLA Class I expression

To determine whether the number of Foxp3+ cells combined with the number of CD8+ cells was related to clinico-pathological parameters or HLA Class I expression, their ratio was calculated. This was performed for both t-Foxp3+ and s-Foxp3+ cell counts. Absolute data on CD8+ cells was only available for the t-CD8+ counts. The ratio t-CD8+/t-Foxp3+ or t-CD8+/s-Foxp3+ could be determined for 63 cases. The median t-CD8+/t-Foxp3+ ratio was 73, median t-CD8+/s-Foxp3+ ratio was 4.03. Due to the distribution of the data the median was used as a cut-off point. Neither of these ratios was related to clinico-pathological parameters or HLA class I expression (data not shown).

Table 2

Presence of Foxp3+ Tregs in tumor compartments related to clinico-pathological characteristics and HLA status

Characteristic	% s-Foxp3+ >median	p-value	% t-Foxp3+ >median	p-value	%t-Foxp3+/ tot- Foxp3+ >median	p-value
Sex (n=76)						
Male	52,3	0.642	47,7	0.941	52,3	0.642
Female	46,9		46,9		46,9	
Age (n=76)						
<65 years	47,4	0.913	52,6	0.910	47,4	0.913
65-70 years	44,4		44,4		44,4	
70-75 years	52,6		42,1		52,6	
>75 years	55		50		55	
Tumor location (n=76)						
Left colon	41,4	0.498	37,9	0.150	41,4	0.410
Right colon	55,2		44,8		51,7	
Rectum	55,6		66,7		61,1	
TNM Stage (n=76)						
I	54,8	0.501	51,6	0.439	45,2	0.437
II	58,3		58,3		66,7	
III	42,4		39,4		48,5	
Differentiation (n=74)						
Poor	46,3	0.505	53,7	0.426	58,5	0.243
Moderate	61,1		38,9		38,9	
Well	42,9		35,7		35,7	
Mucinous (n=69)						
Yes	57,9	0.377	52,6	0.622	57,9	0.377
No	46		46		46	
Jass Classification (n=69)						
Absent-scarce	47,8	0.865	47,8	1.0	56,5	0.395
Moderate-marked	50		47,8		45,7	
MSI status (n=76)						
MSS	46	0.128	46	0.607	49,2	0.761
MSI	69,2		53,8		53,8	
HLA status (n=66)						
Reduced/loss	43,5	0.678	52,2	0.423	65,2	0.03
Expression	48,8		41,9		37,2	

Abbreviation: TNM: Tissue Node Metastasis. MSS: Micro Satellite Stable. MSI: Micro Satellite Instable. HLA: Human Leukocyte Antigen.

Table two shows the relation of either the presence of Foxp3 + cells in the tumor stroma (s-Foxp3+), in the tumor epithelia, (t-Foxp3+) and the ratio t-Foxp3+ / total amount of Foxp3 + (t-Foxp3+/tot-Foxp3+ present to clinico-pathological parameters and HLA expression. Presence is expressed as the percentage of patients that showed levels of s-Foxp3+ and t-Foxp3+ or a ratio above the cut-off level set in all cases at the median

Table 3

Presence of CD8+ CTLs in the various tumor compartments related to the presence of Foxp3 positive cells.

	% s-Foxp3+ >median	p-value	% t-Foxp3+ >median	p-value	%t-Foxp3+/ tot-Foxp3+ >median	p-value
t-CD8+ (n=67)						
Low	46%	0.183	46%	0.621	50%	0.834
High	64.7%		52%		47.1%	
s-CD8+ (n=67)						
no-poor	41%	0.162	46.2%	0.698	51%	0.400
moderate	66.7%		55.6%		55.6%	
marked	60%		40%		30%	
m-CD8+ (n=64)						
no-poor	35.3%	0.043	52%	0.422	52.9%	0.697
moderate	46.7%		40%		53.3%	
marked	76.5%		40.8%		41.2%	

Table three shows the relation of the presence of Foxp3+ cells in the tumor stroma (s-Foxp3+), in the tumor epithelium, (t-Foxp3+) and the ratio t-Foxp3+/ total amount of Foxp3+ cells (t-Foxp3+/ tot-Foxp3+) present to the presence of CD8+ cells in either the tumor epithelium (t-CD8+), the tumor stroma (s-CD8+) or the tumor margin (m-CD8+). The determination and qualification of the presence of CD8+ cells has been previously described. (7)

Association of Foxp3+ cells and the CD8+/Foxp3+ with clinical prognosis

The presence of t-Foxp3+ cells or s-Fop3+ cells was not related to tumor recurrence (*p*-values of 0.598 and 0.446 respectively). Patients with high t-Foxp3+ cell counts compared to patients with low t-Foxp3+ counts showed a trend towards both a longer Overall Survival (OS) time (122 months vs. 96,7 months, *p*=0.084) and a longer Disease Free Survival (DFS) time (109 months vs. 83 months, *p*=0.073). (figure 2&3). Previously, *Menon et al.* have shown that t-CD8+ cells was an independent prognostic factor of a longer disease-free survival. (7)Also the ratio of the number of t-CD8+ cells and number of t -Foxp3+ cells (t-CD8+/t-Foxp3+) showed a trend towards a longer OS time. A ratio above the median, indicating a relative larger number of t-CD8+ cells compared to t-Foxp3+ cells, was related to a longer OS time of 130 months compared to 87 months at a ratio below the median (*p*-value; 0.053). The same trend was seen for DFS, however this was again not significant at a *p*-value of 0.081 (figure 2&3). The t-CD8/s-Foxp3+ ratio was never related to OS or DFS. Interestingly, both the t-CD8+/t-Foxp3+ and t-CD8+/s-Foxp3+ ratio were significantly related to Distant Recurrence Free Survival (DRFS). Both ratios showed a longer DRFS time for patients when above median: 214 months vs. 142 months for the t-CD8+/s-Foxp3+ ratio at a *p*- value of 0.018, and 226 vs. 158 months for the t-CD8+/t-Foxp3+ ratio at a *p*-value of 0.024 (figure 4).

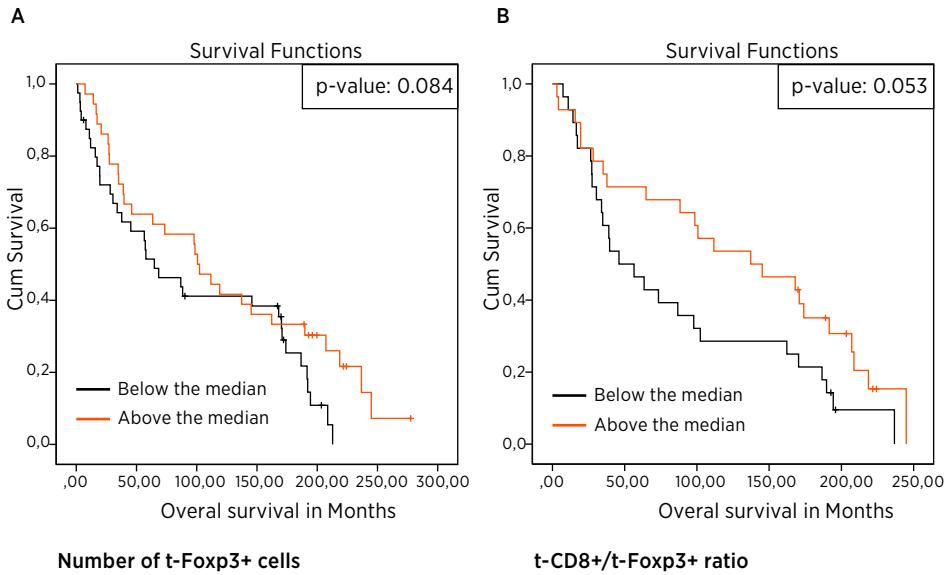


Figure 2

Kaplan Meier graphs for Foxp3 levels in the tumor epithelium (t-Foxp3+) (A) and as a ratio with CD8+ in the tumor epithelium (t-CD8+/t-Foxp3+) (B) in relation to Overall Survival

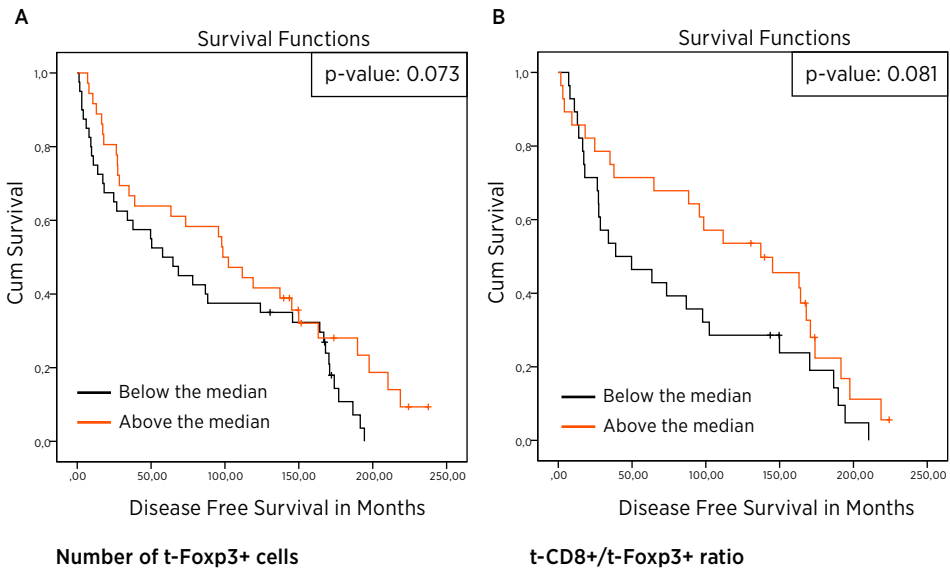


Figure 3

Kaplan Meier graphs for Foxp3 levels in the tumor epithelium (t-Foxp3+) (A) as a ratio with CD8+ in the tumor epithelium (t-CD8+/t-Foxp3+) (B) in relation to Disease Free Survival

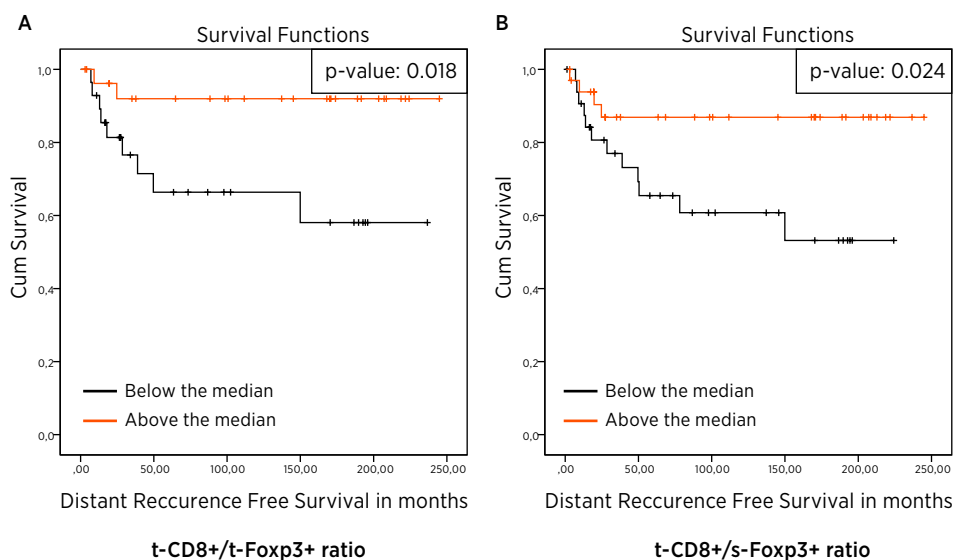


Figure 4

Kaplan Meier graphs for the t-CD8/t-Foxp3 levels (A) and the t-CD8/s-Foxp3+ (B) relation to Distant recurrence Free Survival

Univariate and multivariate Analysis

Uni- and multivariate analysis was performed to calculate hazard ratios, to identify whether the t-CD8+/t-Foxp3+ and t-CD8+/s-Foxp3 ratios were independent of prognostic significance for DRFS. Analysis included known risk factors such as age, TNM stage, tumor microsatellite status, etc. For the DRFS, advanced TNM stage as well as the t-CD8+/t-Foxp3+ and s-Foxp3+ ratios were statistically significant predictors in the univariate analysis. The hazard ratios for TNM stage II and III were 4,294 (CI95% 0,717-25,713) and 8,125 (CI95% 1,847-36,533) respectively at a p-value of 0.003. The hazard ratios of t-CD8+/t-Foxp3+ -and s-Foxp3+ cells were 0.190 (p-value 0.034) and 0.294 (p-value 0.034). However neither of these parameters was able to retain their value as an independent significant predictor of DRFS in multivariate analysis (data not shown). Also for OS analysis, univariate and multivariate analysis were executed with the same parameters. In univariate analysis only mucinous differentiation showed a statistically significant relation to OS. (HR 0.504; CI95% 0.288-0.882; p-value 0.016) This effect did not remain in the multivariate analysis (data not shown)

DISCUSSION

The ability of tumor cells to thrive in a chronically inflamed microenvironment is only one of three important immune system-related hallmarks of tumor growth and development. Two other features also play a major role in the process of tumorigenesis. First, tumors are able to develop certain features that make it possible for them to evade immune recognition.(27) Among others, these features comprise down regulating or even complete loss of HLA class I expression. This way, for the most part they lose the capability to become recognized and attacked by the immune system. Tumor cells may also be poorly immunogenic because of insufficient presence of TAA presentation or co-activating signals. Secondly, tumor cells may evade immune reactivity by restraining an anti-tumor immune response. (5-8) The latter currently gets a lot of attention in tumor immunology as it has become clear that tumor cells may attract immunosuppressive cells such as Foxp3⁺ regulatory T cells (Tregs). Tregs inhibit the development of autoimmune responses, but also are capable of impeding antitumor immune responses. (28;29) In this study we were able to identify and quantify both these features in colorectal tumor samples. In addition we found that both features were seemingly interconnected. The mechanism by which Foxp3⁺ Tregs become attracted to the tumor microenvironment remains yet to be discovered. Two hypotheses can be postulated. Either tumor cells actively attract these cells or the overall inflammatory character induced by tumor growth also induces the influx of Tregs into the tumor microenvironment. Our results showed that down regulation of HLA class I was associated with a relative higher level of epithelial Foxp3⁺ cells as compared to the number of Foxp3⁺ cells in the tumor stromal compartment. This seems to be contradicting, but may be a reflection of an earlier anti-tumor immune reaction. Possibly, tumor reactive CTL caused selective outgrow of HLA loss tumor variances. The immune cells involved, such as the Tregs and CD8⁺ cells, apparently remain present as illustrated by our results.

Our data also illustrate the importance of the interplay between various types of immune cells in the tumor microenvironment in determining clinical outcome as neither the presence of Foxp3⁺ cells nor CD8⁺ cells was statistically significantly prognostic. Combined, however, the ratio of these cells significantly predicted clinical outcome with respect to distant recurrence of disease, suggesting a functional mutual relationship. Previous data published on the effect of Foxp3⁺ cells in CRC patients is rather inconclusive with respect to their prognostic relevance (21-24) One of the reasons for lack of consistency in the results may be variation in study population size as the larger studies by Salama et al. and Suzuki et al. both showed a relationship between Foxp3⁺ cell presence in tumors and clinical prognosis, while studies on smaller populations did not.(22;23) The data presented here, even though derived from a relative small patient cohort, indicates that a more comprehensive approach is probably of greater importance than large patient numbers. This approach, combining data on two types of immune cells that are expected

to interact, but also tumor characteristics that may interfere, will better show the involvement and clinical relevance of the immune system in colorectal cancer. A comparable approach was chosen by Loddenkemper *et al.* and Suzuki *et al.*, but in our study this approach was extended by the incorporation of more data regarding key elements of the process of tumor development. (21;22) Tumor microsatellite status, and other tumor characteristics that were previously shown to be of clinical importance, including HLA class I status, were included in our analysis. The major advantage of this comprehensive ‘mapping’ approach at the level of immune regulation in colorectal cancer is the clinical applicability of the data, as the information might be used to determine treatment allocation, like suggested before. For example, a study by De Kruijf *et al.* showed that the presence of Foxp3+ cells alone in breast cancer patients had no prognostic value.(9) However, when combined with level of HLA expression the data showed a prognostic effect in adjuvant treated patients. It was postulated that counteraction of Tregs by chemotherapy allowed CTL to affect tumor metastases development, but only when HLA expression was intact. (9) Further research conducted in stage III and IV adjuvantly treated patients will determine whether such an effect is also present in CRC patients. In conclusion, we were able to determine a prognostic effect on disease recurrence free survival of the presence of Foxp3+ cells, in either the tumor stromal compartment or the tumor epithelium, but only when combined with data on CD8+ cells. This indicates the importance of a comprehensive approach that shows the interplay between members of the immune system in CRC patients. We hypothesize that studying even more cell types and molecules involved, such as NK ligands, co-stimulating molecules, and activating or suppressing cytokines, will lead to a better understanding of the tumor-immune system interaction. This will eventually help us to better predict patient prognosis and will direct adjuvant treatment.

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CHAPTER 9

Combined analysis of HLA Class I, HLA-E and HLA-G predicts prognosis in colon cancer patients

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British Journal of Cancer. 2014; 110 459-468

ABSTRACT

Background: Evasion of immune surveillance and suppression of the immune system are important hallmarks of tumor development in colon cancer. The goal of this study was to establish a tumor profile based on biomarkers that reflect a tumors' immune susceptibility status and to determine their relation to patient outcome.

Methods: The study population consisted of 285 Stage I-IV colon cancer patients of which a tissue micro array (TMA) was available. Sections were immuno-histochemically stained for presence of Foxp3⁺ cells and tumor expression of HLA Class I (HLA-A, -B, -C) and non-classical HLA-E and HLA-G. All markers were combined for further analyses, resulting in three tumor immune phenotypes: strong immune system tumor recognition, intermediate immune system tumor recognition, and poor immune system tumor recognition.

Results: Loss of HLA class I expression was significantly related to a better OS (p-value 0.005) and DFS (p-value 0.008). Patients with tumors that showed neither HLA class I nor HLA-E or -G expression (phenotype a) had a significant better OS and DFS (p-value <0.001 and 0.001, respectively) compared to phenotype b (OS HR 4.7, 95% CI 1.2-19.0, p=0.001) or c (OS HR 8.2, 95% CI 2.0-34.2, p=0.0001). Furthermore, the tumor immune phenotype was an independent predictor for OS and DFS (p-value 0.009 and 0.013 respectively).

Conclusions: Tumors showing absence of HLA class I, HLA-E and HLA-G expression were related to a better OS and DFS. By combining the expression status of several immune-related biomarkers, three tumor immune phenotypes were created that related to patient outcome. These immune phenotypes represented significant, independent, clinical prognostic profiles in colon cancer.

INTRODUCTION

Historically, the immune system has been attributed an important role in controlling tumor growth and metastasis (1–4). Evasion of immune surveillance and suppression of the immune system are two important traits cancer cells have to acquire during the process of tumorigenesis (5). Research of the last century has indicated that the influence of the immune system on tumor cells, both in the tumor micro-environment as well as during the process of tumor metastasis, also contributes to tumor progression (6). The cancer immune-editing hypothesis describes both the host-protective as well as the tumor-promoting actions the immune system might have on developing tumors, shaping tumor immunogenicity. (7–13). Tumors are thought to be ‘edited’ through a Darwinian selection process into poorly immunogenic tumor cell variants invisible to the immune system and able to grow progressively. Immune-editing might therefore have substantial effects on patient’s prognosis.

Several mechanisms taking place at the tumor cell level contribute to this process. The first mechanism is downregulation of human leukocyte antigen (HLA) class I expression. Downregulation of HLA class I minimizes the level of tumor-associated antigen (TAA) expression by tumor cells and therefore their recognition and subsequently destruction by cytotoxic T-cells (CTL) (5,14–16). The second mechanism is the ability of tumor cells to regulate the expression of non-classical HLA class I molecules (HLA-E and HLA-G) on the cell surface. Expression of these markers has been found to inhibit Natural Killer (NK) cell recognition in the blood stream and therefore results in further tumor cell escape from immune surveillance (17–20). HLA-E is regularly expressed in various healthy tissues and correlated with HLA class I expression (21). In contrast, HLA-G is rarely found in healthy tissues, but is frequently observed in tumors (19). Thirdly, tumor cell immune reactivity can become suppressed by the attraction of immunosuppressive regulatory T cells (Tregs) into the tumor micro-environment (22,23). Tregs are able to modulate the anti-tumor immune response as they suppress the activity of CTL through direct cell-to-cell contact or via the release of cytokines like transforming growth factor β (24–26). Tregs and CTLs therefore show opposing actions in tumor immunity (27). Previously, both the downregulation of HLA class I, presence of Tregs and HLA-E and -G expression have been shown to be of clinical relevance in several types of cancers (28–31). In colorectal cancer (CRC), various studies have described the impact of the level of HLA class I tumor expression or the presence of Foxp3+ Tregs cells on patients with varying results (32–39). In general, loss of HLA class I tumor expression seemed to result in a better prognosis (39,40). The presence of high levels of Foxp3+ cells in CRC patients was related to a worse prognosis in some studies, although this relation could not always be established in CRC patients (33,34,37,38,41). Studies on the prognostic value of HLA-E and HLA-G showed that expression of these molecules correlated with poor prognosis and tumor progression (42–45).

Previous studies have shown a complex interaction between different immune markers, highlighting the need for combined marker analysis (29,41,46). The purpose of this study was to investigate the prognostic value of the immune-related biomarkers HLA Class I, HLA-E and -G and Foxp3+, to establish distinct patterns that reflect a tumor's immune-escape mechanism by combining these markers, and to relate these patterns to clinical outcome.

MATERIALS AND METHODS

Study population

The patient population comprised a consecutive series of 470 colorectal cancer patients all treated with surgery for their primary tumor in the Leiden University Medical Center (LUMC) between 1991 and 2001. Of these patients tumor material, clinico-pathological data and information on the follow-up was collected in retrospect. This research was approved by the Medical Ethical Committee of the LUMC. Mucinous differentiation was defined as fully (>50%), partly (0-50%) or no mucinous differentiation. Tumor Node Metastasis (TNM) was defined by the Union for International Cancer Control (UICC) (47). Tumor differentiation was defined as good, moderate or poor, as described in the pathology report. Patients with rectal cancer, patients with a history of cancer other than basal cell carcinoma or cervical carcinoma *in situ*, patients with more than one colon tumor at the same time, and patients that received radio- or chemotherapy treatment prior to resection were excluded from the analysis (n=185 in total). The study cohort therefore consisted of 285 colon cancer patients.

Antibodies

The mouse monoclonal antibodies HCA2 and HC10 were used, which recognize the heavy chains of HLA Class I, and were kindly provided by Prof. Dr. J. Neefjes. The reactivity spectrum of HCA2 comprises all HLA-A chains (except HLA-A24), as well as some HLA-B, HLA-C, HLA-E, HLA-F, and HLA-G chains. HC10 reacts with HLA-B and HLA-C heavy chains and some HLA-A (HLA-A10, HLA-A28, HLA-A29, HLA-A30, HLA-A31, HLA-A32, HLA-A33) (46). The mouse antibodies against human Foxp3 (ab20034 clone 236A/E7; Abcam) were used for Treg identification. The reactivity spectrum of Foxp3 is composed of regulatory T cells and may include small numbers of CD8+ cells but is generally considered to be the best single marker for Treg identification (48,49). For HLA-E and HLA-G identification mouse monoclonal antibodies against HLA-E (ab2216 clone MEM-E/02: AbCam, UK) and HLA-G (4H84: Exbio, Czech Republic) were used. MEM-E/02 recognizes denatured HLA-E (50,51), while 4H84 recognizes denatured HLA-G molecules and also binds to free heavy chains of classical HLA class I molecules (51-53).

TMA production and immunohistochemistry

The histo-pathological characteristics of the tumor material from all patients included were determined by qualified pathologists according to current standards. Of the formalin-fixed paraffin-embedded (FFPE) tumor blocks of the primary tumors, sections were cut for haematoxylin and eosin staining. Based on microscopic inspection of the slides, histo-pathologically representative bulk tumor regions from each tumor block were identified and punched for preparation of tumor tissue microarray (TMA) blocks. From each donor block, three 0.6 mm diameter tissue cores were punched from the identified tumor areas and transferred into a receiver paraffin block using a custom-made precision instrument. Immuno-histochemical staining (IHC) for Foxp3+ cells, non-classical HLA-E and HLA-G, and classical HLA class I tumor expression was performed on 4 µm sections, which were cut from each receiver block and mounted on glass.

The sections were deparaffinized and rehydrated. Endogenous peroxidase was blocked for 20 minutes in 0.3% hydrogen peroxide in PBS. For antigen retrieval, slides were boiled in 0.01 M EDTA buffer (pH 8) for 10 minutes at maximum power in a microwave oven. Sections were incubated overnight with anti-Foxp3+, -HLA-E or -HLA-G antibodies at pre-determined optimal dilution. After 30 minutes of incubation with Envision anti-mouse (K4001; DAKO Cytomation, Glostrup, Denmark), sections were visualized using diaminobenzidine solution (DAB+). Tissue sections were counterstained with haematoxylin, dehydrated and finally mounted in pertex. The IHC for HCA2 and HC10 was performed using the Autostainer Link 48 (DAKO). For antigen retrieval Envision TM Target Retrieval Solution (DAKO), pH low, was used. The sections were incubated for 18 hours with either HCA2 or HC10 antibodies at pre-determined optimal dilution, followed by incubation with Envision FLEX/HRP (DAKO). Sections were visualized using DAB+ liquid solution (DAKO). Finally these slides were counterstained with haematoxylin as well, dehydrated and finally mounted in pertex. All slides were stained simultaneously to avoid interassay variation. For each patient, normal epithelium, stromal cells, or lymphoid cells served as internal positive control for HLA class I antibody reactivity. Placenta tissue slides served as positive control for HLA-E and HLA-G staining. Slides from human tonsil tissue served as positive control for Foxp3+ staining. Negative controls were tissue slides that did undergo the whole immunohistochemical staining without primary antibody.

Evaluation of immunohistochemistry

Microscopic analysis of HCA2, HC10, HLA-E and HLA-G expression and presence of Foxp3 + cells was performed by two independent observers in a blinded manner (M.S.R.: 100% of the cohort, E.C.M.Z. 30% of the cohort). The Cohen's Kappa was > 0.75 for all stainings indicating substantial agreement between the two observers. The scores of the three 0.6 mm punches were averaged. For HCA2 and HC10 the percentage of tumor cells with membranous staining was assessed. HLA class I expression status was determined according to the standard set by the International HLA and Immunogenetics Workshop (54). HCA2 and HC10 expression

percentages were divided into two categories; 0-5% of the tumor cells show expression and 5-100% show expression. If <5% of the tumor cells showed expression for each of the two markers, this was determined to represent loss of HLA class I expression; if expression in <5% of the tumor cells of one of the two markers as HLA class I downregulation; and if expression in more than 5% of the tumor cells for each of the two markers this was denoted as HLA class I expression. For HLA-E and HLA-G, intensity of tumor staining (absent, weak, moderate or strong intensity) was determined. For HLA-E, absent and weak staining together versus moderate and strong staining together were used for the final analysis. For HLA-G, absent tumor staining was analyzed versus weak, moderate and strong tumor staining together, because HLA-G is normally not expressed on healthy tissues in comparison to HLA-E (19,21). Quantification of the number of Foxp3⁺ cells was microscopically assessed in the entire tumor punches of the TMA and the absolute number of positive cells was used for the analysis.

Determination of microsatellite stability status

DNA was extracted from 2mm tumor-cores. Paraffin was dissolved in xylene, tissue was rehydrated in ethanol (100%/70%) and dried for 10 minutes at 37°C. Nucleospin 96 Tissue kit (Machery-Nagel, Düren, Germany) was used for DNA extraction according to the manufacturer's protocol.

MSS-status was tested using the MSI Analysis System Version 1.2 (Promega, Mannheim, Germany) and interpreted by an experienced pathologist, as described previously (55).

Statistical Analysis

Statistical analyses were performed using the statistical package SPSS (version 17.0 for Windows; SPSS Inc.). The Student's T-test and the Chi-squared test were used to evaluate associations between tumor expressions of HLA class I, and non-classical HLA-E and HLA-G and tumor infiltration of Foxp3⁺ cells and various clinico-pathological variables. Overall Survival (OS) was defined as time of surgery until death and Disease Free Survival (DFS) as time of surgery until death or relapse of disease, whichever came first. The Kaplan-Meier method was used for calculation of survival probabilities and the Log-rank test for comparison of survival curves between these three phenotypes. Cox regression was used for univariate and multivariate analysis for OS and DFS. Significant variables ($p < 0.05$) in univariate analysis were included in multivariate analysis.

RESULTS

HLA class I expression

Microscopic quantification of HLA class I expression was performed on 242 patients as, due to staining artifacts and loss of material during the staining procedure, the IHC results of 43 cases could not be analyzed. Representative images of HLA class I staining and frequencies of HLA class I expression in the different groups are shown in figure 1 and 2. Patient characteristics and data on HLA class I expression are shown in table 1. Since HCA2 also reacts with some HLA-G chains (46), we examined the relationship between HCA-2 reactivity and HLA-G expression and found no correlation ($p=0.348$).

Patients whose tumors showed loss of HLA class I had a significantly better Overall Survival (OS) and Disease Free Survival (DFS) (logrank p-value 0.005 and 0.008) compared to patients with tumors with HLA class I downregulation or expression (figure 3). The Hazard Ratios (HRs) for OS and DFS for HLA class I tumor expression are shown in Table IIa and IIb

Foxp3+ cells

The number of Foxp3+ cells could be evaluated in 245 patients, because, due to staining artifacts and loss of material during the staining procedure, the IHC results of 40 cases could not be analyzed. The mean number of positive cells per tumor punch was 19 with a median of 12.0. In 4.1% ($n=10$) of the patients no Foxp3+ cells were present. Representative images of Foxp3+ staining are shown in figure 1. Patients with expression of HLA class I showed borderline significantly higher levels of Foxp3+ cells in their tumor punches compared to HLA class I downregulation or loss: mean in expression group 21 vs. a mean of 12 and 14 positive cells in the downregulation group and loss of HLA class I group respectively; p-value 0.07. Patients with stage 1 tumors showed significantly higher levels of Foxp3+ cells compared to patients with stage 2, stage 3 and stage 4 tumors: mean level of Foxp3+ cells in stage 1 tumors was 38 compared to 13, 17 and 20 for the stage 2, 3 and 4 tumors, p-value <0.001 . For further analysis Foxp3+ was categorized as below vs. above median based on the median due to the skewness in the spread of the data. Frequencies are shown in figure 2. The presence of Foxp3+ cells in the tumor micro-environment was not related to OS (logrank p-value 0.114) or DFS (logrank p-value 0.155).

HLA-E and HLA-G

Representative images for HLA-E and HLA-G and frequencies in the different groups are shown in figure 1 and 2. HLA-E and HLA-G were not related to OS and DFS (logrank p-values for OS 0.809 and 0.239 respectively, logrank p-values for DFS 0.876 and 0.117 respectively). None of the clinico-pathological characteristics were significantly related to tumor expression of HLA-E or HLA-G (data not shown).

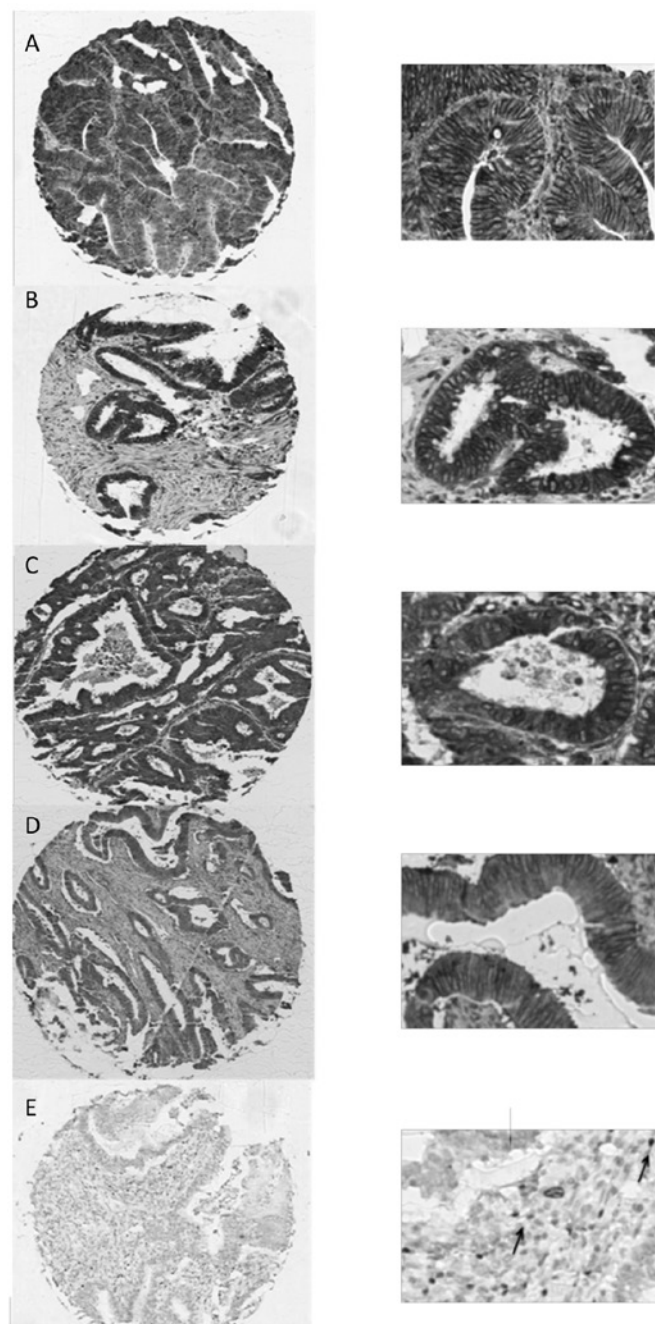


Figure 1

Representative images of HLA class I, HLA-E, HLA-G and Foxp3+ staining.

Representative images of immuno-histochemical stainings for HLA Class I expression (HCA2 and HC10), HLA-E and HLA-G expression and presence of FOXP3+ on the left side with magnifications on the right side, performed according to standard protocols (details in *Material and Methods*).

(A) HCA2-positive tumor (B) HC10-positive tumor (C) HLA-E-positive tumor (D) HLA-G- positive tumor and (E) Presence of Foxp3+ cells as indicated by the arrows.

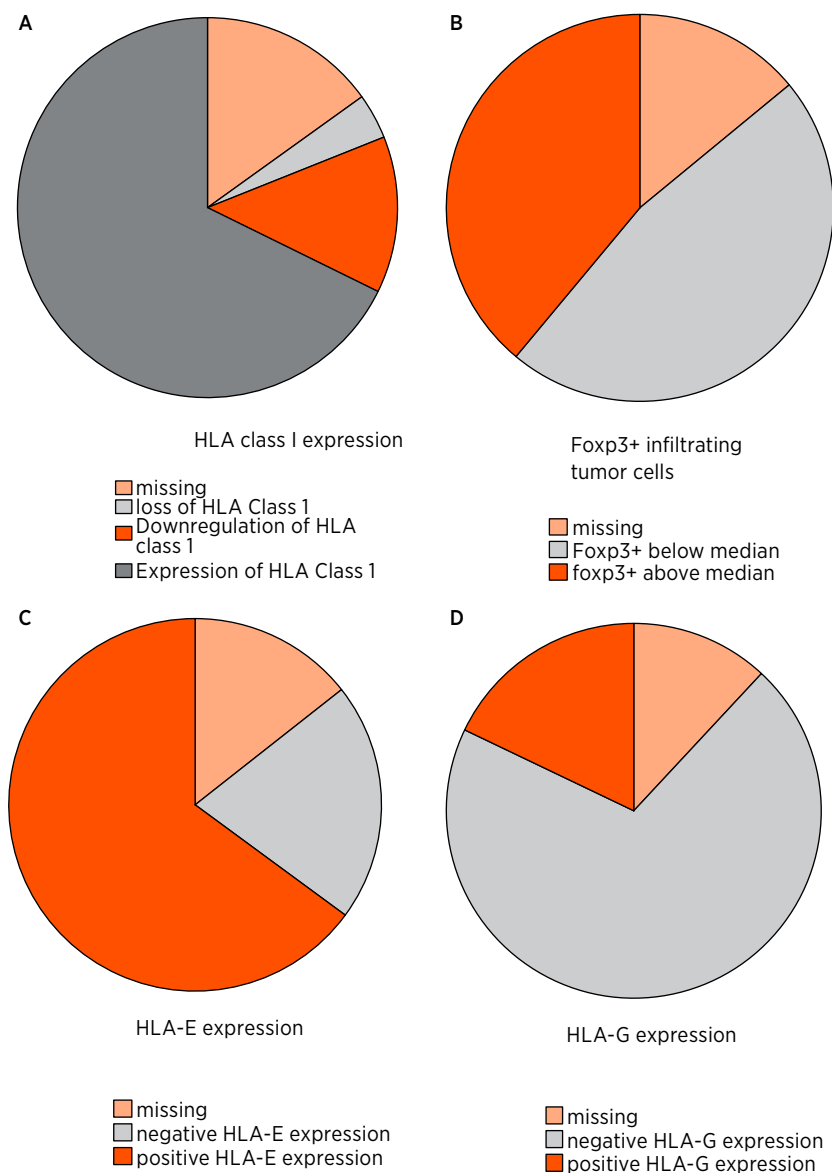


Figure 2

Frequencies of HLA class I tumor expression, Foxp3+ tumor infiltration and HLA-E and -G tumor expression. Pie-charts indicating the frequencies of all stainings including missings due to staining artifacts and loss during the staining procedure. Details about group composition and scoring methods are written in *Material and Methods* (A) Frequency of HLA class I tumor expression; missing 43/285 (15.1%), loss of HLA class 1 11/242 (4.5%), Downregulation of HLA class 1 38/242 (15.7%), Expression of HLA Class 1 193/242 (79.8%) (B) Frequency of Foxp3+ tumor cell infiltration; Foxp3+ below median 134/245 (54.7%) Foxp3+ above median 111/245 (45.3%) Missing 40/285 (14.0%). (C) Frequency of HLA-E tumor expression. Negative HLA-E expression 59/244 (24.2%) Positive HLA-E expression 185/244 (75.8%) Missing 41/285 (14.4%) (D) Frequency of HLA-G tumor expression. Negative HLA-G expression 200/251 (79.9%) Positive HLA-G expression 51/251 (20.3%) Missing 34/285 (11.9%)

Table I

Patient Characteristics of the Total Colon Cancer Cohort and stratified for H+LA class I, HLA-EG and Foxp3+ expression

	Total pop- ulation (n=285)	HLA Class I Loss (n=11)	HLA Class I Down- regulation (n=38)	HLA Class I Expression (n=193)	HLA-EG Absence (n=202)	HLA-EG Presence (n=42)	Foxp3+ Absence (n=134)	Foxp3+ Presence (n=111)
Gender (%)								
Male	137(48.1)	6 (54.5)	18 (47.4)	99 (51.3)	103 (51.0)	17 (41.5)	63 (47.0)	57 (51.4)
Female	148 (51.9)	5 (45.5)	20 (52.6)	94 (48.3)	99 (49.0)	25 (58.5)	71 (53.0)	54 (48.6)
Age in years (%)								
Below 50	32 (11.3)	3 (27.3)	1 (2.6)	23 (12.0)	24 (12.0)	3 (7.1)	13 (9.7)	14 (12.8)
Above 50	251 (88.7)	8 (72.9)	37 (97.4)	168 (88.0)	176 (88.0)	39 (92.9)	121 (90.3)	95 (87.2)
T stage (%)								<0.001
1	17 (6.0)	0	2 (5.3)	12 (6.3)	11 (5.5)	3 (7.1)	3 (2.3)	11 (9.9)
2	37 (13.0)	1 (9.1)	1 (2.6)	25 (13.0)	24 (11.9)	4 (9.5)	7 (5.3)	21 (18.9)
3	193 (68.0)	8 (72.7)	26 (68.4)	135 (70.3)	143 (71.1)	27 (64.3)	104 (78.2)	67 (60.4)
4	37 (13.0)	2 (18.2)	9 (23.7)	20 (10.4)	23 (11.4)	8 (19.0)	19 (14.3)	12 (10.8)
Differentia- tion (%)								
Moderate	145 (64.2)	4 (44.4)	16 (64.0)	104 (65.4)	104 (64.2)	20 (64.5)	69 (64.5)	56 (64.4)
Poor	23 (10.2)	0 (0)	3 (12.0)	18 (11.3)	15 (9.3)	6 (19.4)	11 (10.3)	10 (11.5)
Good	58 (25.7)	5 (55.6)	6 (24.0)	37 (23.3)	43 (26.5)	5 (16.1)	27 (25.2)	21 (24.1)
Mucinous aspect(%)								
No	233 (83.5)	7 (63.6)	28 (77.8)	165 (86.8)	164 (83.2)	36 (85.7)	105 (80.2)	96 (88.1)
Fully	33(11.8)	4 (36.4)	6 (16.7)	15 (7.9)	24 (12.2)	3 (7.1)	18 (13.7)	9 (8.3)
Partly	13 (4.6)	0 (0)	2 (5.6)	10 (5.3)	9 (4.6)	3 (7.1)	8 (6.1)	4 (3.7)
Microsatel- lite stabil- ity(%)								
MSS	168 (84.8)	6 (66.7)	25 (86.2)	132 (87.4)	136 (86.6)	29 (85.3)	84 (81.6)	81 (92.0)
MSI	30 (15.2)	3 (33.3)	4 (13.8)	19 (12.6)	21 (13.4)	5 (14.7)	19 (18.4)	7 (8.0)

Abbreviations. MSS; microsatellite stability, MSI; Microsatellite instability.

Note: HLA-EG is a combination of HLA-E and HLA-G (explained in the material and methods section).

This table shows the patient characteristics of the entire colon cancer cohort (n=285) and stratified according to HLA class I, HLA-EG and Foxp3+ staining. Only T stage was significantly related to Foxp3+.

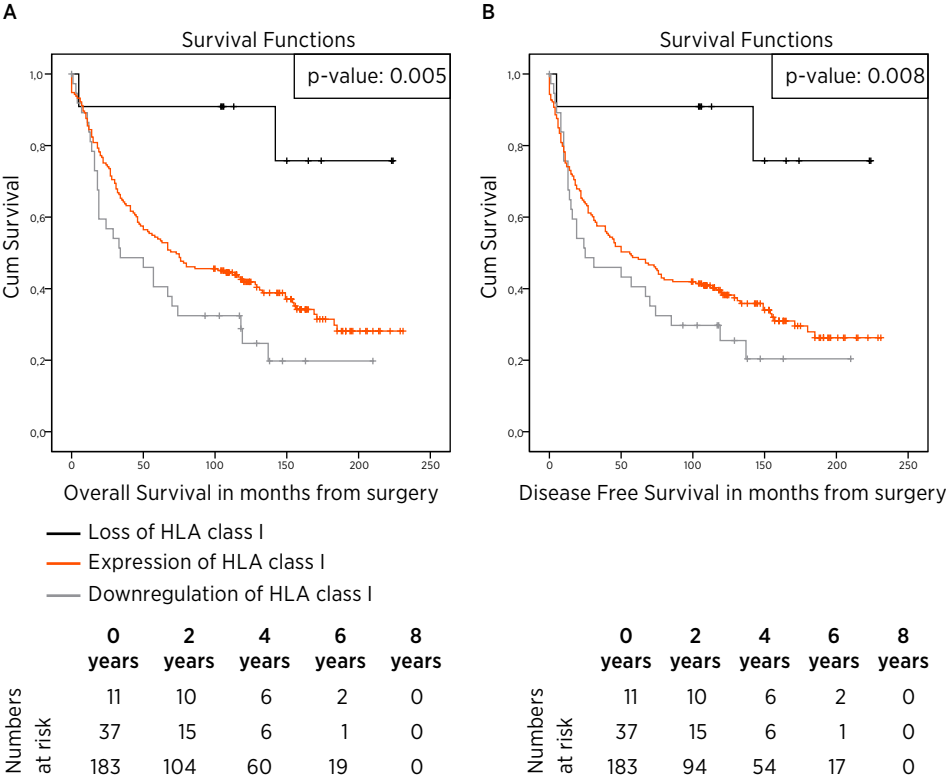


Figure 3
Survival curves stratified for HLA class I tumor expression in colon cancer
A) Kaplan Meier curve for OS in the study population of 285 colon cancer patients stratified for HLA class I tumor expression status. B) Kaplan Meier curve for DFS in the study population of 285 CRC patients again stratified for HLA Class I tumor expression.

A combined variable of HLA-E and HLA-G scores was created (cEG). Expression was considered positive when both HLA-E and HLA-G were expressed (cEG+) and negative when either HLA-E or HLA-G was not expressed (cEG-). The group with tumors with both absences of HLA-E and HLA-G was too small to analyze separately. Positive cEG was found in 14.7% (42 of 244) of tumors. Patient characteristics and data on the combined variable HLA-E and -G expression can be found in table 1. None of the clinico-pathological variables shown in table 1 were significantly related to tumor expression of cEG. cEG was not significantly related to OS (logrank p-value 0.245) and DFS (logrank p-value 0.100).

Multivariate analysis for single immune markers

Both for OS and DFS a univariate analysis was performed for the following parameters: sex, age, TNM stage, HLA class I expression status, mucinous differentiation, tumor grade, adjuvant therapy and microsatellite status. In the univariate analysis for OS, age (p-value <0.001), TNM status (p-value <0.001) and HLA class I expression status (p-value 0.011) were significant predictors of survival. The same was true for the univariate analysis for DFS with a p-value of <0.001 for age and TNM status and a p-value of 0.02 for HLA class I expression. Therefore all three were included in the multivariate analysis. In this analysis age and TNM stage remained significant for both OS and DFS (OS and DFS p-values all <0.001): HLA class I was a borderline independent significant predictor for OS (p-value 0.08) (table IIa and IIb).

Analysis of tumor immune phenotypes

Except for HLA class I, none of the tumor immune markers showed a significant correlation with patients' clinical outcome. The interaction between tumor cells and immune cells, however, is complex and multifaceted. Therefore, we hypothesized that analysis of combined tumor immune markers; describing a tumor's immune phenotype may better reflect outcome of the interaction between tumor cells and the immune system. We combined all of the data into one combined variable. The Kaplan Meier curves performed with this combined variable indeed revealed 3 distinct patterns in relation to patient outcome (figure 4 and 5). The entire population could be divided in 3 phenotypes:

- a) Strong immune system tumor recognition: Patients with tumors that showed loss of HLA class I expression, presence of Foxp3+ cells in the tumor micro-environment, and negative cEG expression (n=11).
- b) Intermediate immune system tumor recognition: Patients with tumors that showed downregulation of HLA Class I expression and negative cEG expression, but were found to have Foxp3+ cells in the tumor micro-environment or patients with tumors that showed normal HLA class I expression irrespective of cEG expression and the presence of Foxp3+ cells (n=184).
- c) Poor immune system tumor recognition: Patients with tumors showing normal or downregulated HLA class I and no presence of Foxp3+ cells irrespective of their cEG expression (n=460).

Table IIa

Univariate and multivariate analyses of Overall Survival (OS) in the different immune markers and in the tumor immune phenotypes

	Univariate analysis			Multivariate analysis*		
	HR	95% CI	p-value	HR	95% CI	p-value
HLA class I			0.011			0.08
Loss	1.0			1.0		
Downregulation	7.3	1.7-30.8		4.3	1.0-18.5	
Expression	4.9	1.2-20.0		3.1	0.7-12.6	
Foxp3+			0.116			
Below median	1.0					
Above median	0.8	0.6-1.1				
HLA-E			0.810			
Negative	1.0					
Positive	1.0	0.7-1.4				
HLA-G			0.242			
Negative	1.0					
Positive	1.2	0.9-1.8				
HLA-EG			0.248			
Negative	1.0					
Positive	1.3	0.8-1.9				
Immune phenotypes			0.001			0.009
Phenotype a	1.0			1.0		
Phenotype b	4.7	1.2-19.0		2.9	0.7-11.9	
Phenotype c	8.2	2.0-34.2		4.8	1.1-20.2	

*Corrected for sex, age, TNM stage, HLA class I expression status, mucinous differentiation, tumor grade, adjuvant therapy and microsatellite status. Only significant variables in univariate analysis are corrected in multivariate analysis. Note: HLA-EG is a combination of HLA-E and HLA-G (as explained in the results section).

These three phenotypes showed significant differences for OS (logrank p-value <0.001) and DFS (logrank p-value 0.001). The HRs of the three phenotypes for OS and DFS are shown in table IIa and IIb.

Multivariate analysis for tumor immune phenotypes

Again, both for OS and DFS a univariate analysis was performed for the following parameters: sex, age, TNM stage, tumor immune phenotype, mucinous differentiation, tumor grade, adjuvant therapy, and microsatellite status. In univariate analysis, next to age and TNM status, the tumor immune phenotype was a significant predictor for OS (p-value 0.001) and DFS (p-value 0.002). Therefore all three these parameters were included in multivariate analysis. The tumor immune phenotype was an independent significant predictor for both OS (p-value 0.009) and DFS (p-value 0.013) and HRs are shown in table IIa and IIb.

Table IIb

Univariate and multivariate analyses of Disease Free Survival (DFS) in the different immune markers and in the tumor immune phenotypes

	Univariate analysis			Multivariate analysis*		
	HR	95% CI	p-value	HR	95% CI	p-value
HLA class I			0.021			0.104
Loss	1.00			1.00		
Downregulation	7.2	1.7-30.1		4.6	1.1-19.7	
Expression	5.4	1.3-21.8		3.7	0.9-15.0	
Foxp3+			0.159			
Below median	1.00					
Above median	0.8	0.6-1.1				
HLA-E			0.877			
Negative	1.00					
Positive	0.97	0.7-1.4				
HLA-G			0.121			
Negative	1.00					
Positive	1.3	0.9-1.9				
HLA-EG			0.104			
Negative	1.00					
Positive	1.4	0.9-2.1				
Immune phenotypes			0.002			0.013
Phenotype a	1.00			1.00		
Phenotype b	5.1	1.3-20.7		3.5	0.8-14.2	
Phenotype c	8.4	2.0-34.9		5.4	1.3-22.7	

*Corrected for sex, age, TNM stage, HLA class I expression status, mucinous differentiation, tumor grade, adjuvant therapy and microsatellite status. Only significant variables in univariate analysis are corrected in multivariate analysis. Note: HLA-EG is a combination of HLA-E and HLA-G (as explained in the results section).

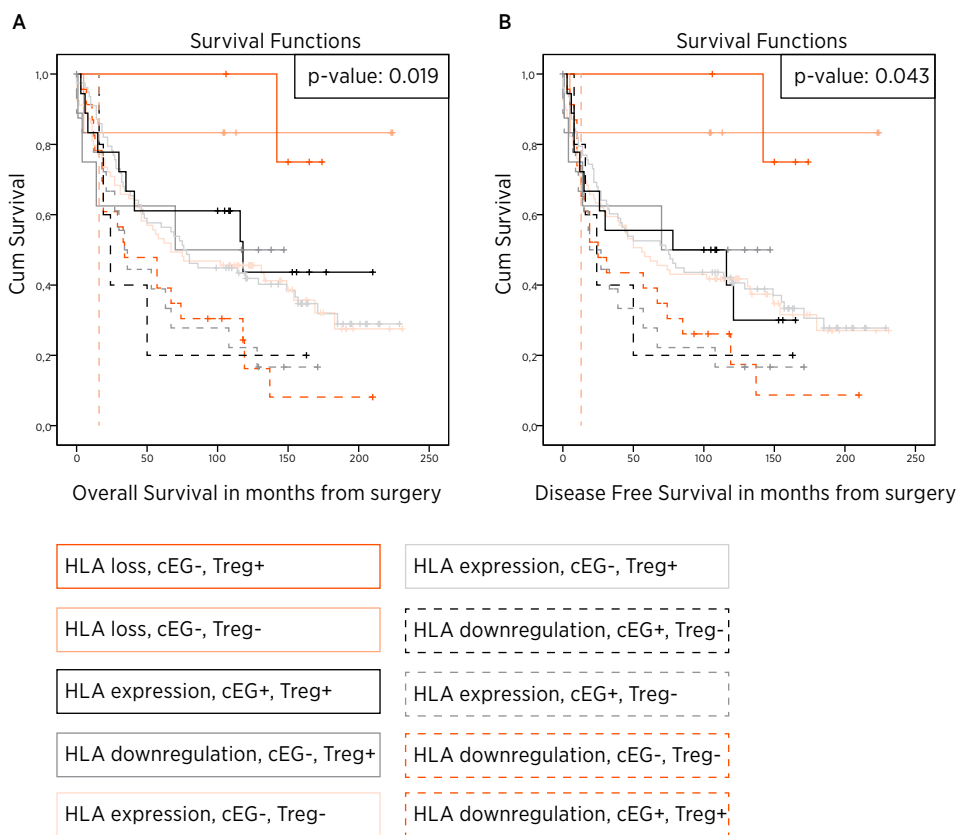


Figure 4

Survival curves stratified for combined tumor expression of HLA class I, HLA-E, HLA-G and Foxp3+ in colon cancer. A) Kaplan Meier curve for OS in the study population of 285 colon cancer patients stratified for all the different combinations between tumor expression of HLA class I, combined expression of HLA-E and HLA-G (cEG) and the presence of Foxp3+ cells (Tregs) based on which 3 distinct patterns could be distinguished as shown in figure 4 B) Kaplan Meier curve for DFS in the study population of 285 colon cancer patients stratified for all the different combinations between tumor expression of HLA class I, combined expression of HLA-E and HLA-G (cEG) and the presence of Foxp3+ cells (Tregs) based on which 3 distinct patterns could be distinguished as shown in figure 4.

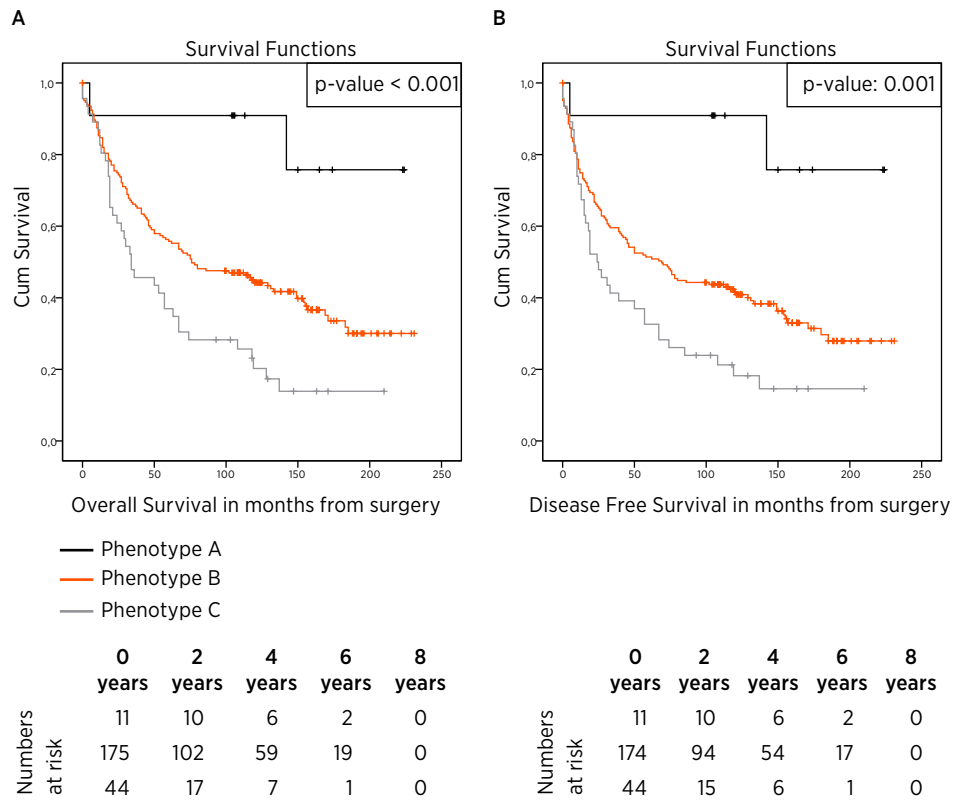


Figure 5

Survival curves stratified for immune phenotypes in colon cancer.

A) Kaplan Meier curve for OS in the study population of 285 colon cancer patients stratified for all the different combinations between tumor expression of HLA class I, combined expression of HLA-E and HLA-G (cEG) and the presence of Foxp3+ cells (Tregs) based on which 3 immune phenotypes could be distinguished. See results section for explanation of the phenotypes. B) Kaplan Meier curve for DFS in the study population of 285 colon cancer patients stratified for all the different combinations between tumor expression of HLA class I, combined expression of HLA-E and HLA-G (cEG) and the presence of Foxp3+ cells (Tregs) based on which 3 immune phenotypes could be distinguished. See results section for explanation of the phenotypes.

DISCUSSION

Tumor-immune interactions may be important for the prognosis of cancer patients (17). In this study, we showed that by combining the immune-related markers HLA class I, HLA-E, HLA-G and Foxp3+, we were able to determine three distinct patterns in survival, which might represent how immune surveillance controls tumor growth and metastasis.

The first marker of tumor-immunogenicity used was the level of HLA class I expression of cancer cells. Our results are comparable with the results of other studies that were able to determine a prognostic effect of the HLA class I status in colon cancer (35,39). Watson *et al.* showed that tumors with downregulation of HLA class I had a worse survival comparable with our results (39). In contrast, Menon *et al.* showed a survival benefit in patients with downregulated HLA-A tumors (35). However, when HLA-A and HLA-B/C were combined, statistical significance was lost. Furthermore, patients with expression of HLA class I were related to a better survival in the study by Watson *et al.*, whereas our study and the study by Menon *et al.* showed an improved survival in patients with loss of HLA class I expression. Possible explanations for these differences might be a different definition for HLA class I expression, differences in staining techniques and scoring, a different patient cohort and microsatellite instability (MSI), which is associated with loss of HLA class I and a better prognosis (56,57). In our study, 33% of the tumors with loss of HLA class I showed the MSI phenotype, in comparison to 14% and 13% for HLA class I downregulation and expression. Results from Menon *et al.* also showed that 50% of the tumors with loss of HLA class I had the MSI phenotype. In contrast, Watson *et al.* did not mention MSI.

As hypothesized, loss of HLA class I expression in tumor cells could also be related to a better patient survival because such cells, once they metastasize to the bloodstream, are eliminated by NK cell attacks (35,39,58). Tumors with loss of HLA class I have also shown to have significantly higher NK cell infiltration (15). More interestingly, the tumors showing loss of HLA class I in our cohort were also the ones that showed to be negative for HLA-E and -G expression (phenotype a). Absence of the HLA-E and -G expression makes them even more susceptible to NK cell elimination (17-20). Furthermore, this is also confirmed by CRC tumors with loss of HLA class I expression who do not metastasize to the liver (59).

The presence of the third marker Foxp3+ is thought to represent the inhibition of host-protective antitumor responses. When stimulated, they inhibit the function of CTL (6). Although the exact mechanism by which these cells are drawn into the tumor micro-environment remains unexplained, their immunosuppressive effect has been proven with a high density of tumor-infiltrating Foxp3+ cells found to be associated with an unfavorable prognosis in a wide range of human carcinomas, including breast and lung cancer (60,61). However, in colon cancer different results are reported as well (37,38). One possible explanation for these opposite results might be a different micro-environment of colon cancer, which is colonized with many gastro-intestinal bacteria, triggering the production of pro-inflammatory

cytokines causing tumor-enhancing effects. Instead of the specificity of infiltrating T-cells for tumor-antigens, T-cells in colon cancer could be more specific for the microflora and suppress inflammation and immune responses from bacterial invasion, resulting in an anti-tumorigenic effect, which could explain the better prognosis of patients with tumors with a strong Foxp3+ infiltration (62). We were not able to demonstrate differences in disease outcome for Foxp3+ tumor infiltration supporting this latter hypothesis, but we did see differences in Foxp3+ infiltration if we combined them with HLA class I expression and with HLA-E and -G expression, especially in patients who have retained their HLA class I expression. Patients with normal HLA class I expression and absence of Foxp3+ cell infiltration showed a worse patient outcome. We hypothesize that the tumors of these patients have had a minimal CTL attack because the HLA class I expression is preserved. Since CTL and Foxp3+ cells show opposing actions (27) and CTLs are possibly scarce in these tumors, Foxp3+ cell infiltration might not be necessary. These tumors could therefore progress aggressively as immune surveillance is poor. In contrast, tumors with HLA class I expression, which were able to attract Foxp3+ cells, showed a slightly better prognosis. In this case, Foxp3+ cell infiltration might indicate CTL activity resulting in suppression of the tumor growth.

Therefore, in our opinion, the clinical relevance of the studies by Watson *et al.* and several others does not provide an optimal perspective on prognosis (35,39), because expression of a single immune marker is not sufficient for the selection of high-risk colon cancer patients or treatment allocation. As shown by our results and previous studies, immune markers are related to each other (29,46,63,64).

When all markers were combined, patients showing the worst prognosis were patients with HLA class I downregulation, negative or positive cEG expression and absence of Foxp3+ cells denoted as phenotype c. We hypothesize that these poor immune system recognized tumors were able to elicit only a minimal CTL attack because they partly preserved HLA class I expression and subsequently attracted little to no Foxp3+ cells in their tumor micro-environment. Furthermore, these tumors showed a positive expression of HLA-E and -G, further escaping immune surveillance through inhibition of NK cell recognition (17-20). These tumor cells can therefore quickly progress to the bloodstream and might eventually metastasize.

It is important to realize that what we are evaluating is just a 'snapshot' of the ongoing process of cancer immuno-editing in the patient's primary tumor at time of resection. Still, from a clinical point of view, at the patient's bedside this is usually the only data available based on which clinical decision making has to take place and these data can actually be of clinical value to, for example, the allocation of adjuvant therapy as opposed by De Kruif *et al.* in breast cancer and other studies (29,65,66).

Our study does have a few limitations. Not all combinations between HLA class I, HLA-E and -G and Foxp3+ were present in our cohort. There was no representation of tumors with loss of HLA class I, which were HLA-E and -G positive. Therefore we were not able to investigate the prognosis of these tumors, but we hypothesize that these tumors have a worse prognosis as these tumors might escape

NK cell attack. Although there is a physiological correlation between HLA-E and HLA class I molecules, this has been found to be disturbed in tumors, suggesting further escape from immune recognition through upregulation of HLA-E (21,46). To truly investigate these tumors, our study has to be validated in a bigger cohort. Second, the antibodies we used for HLA class I detection only detected the heavy chain, but not the trimeric complex consisting of β 2-microglobuline heavy chain and antigen (67). Therefore we should be careful using the term total loss of HLA class I. Third, we did not investigate the role of NK cells in patients with loss or downregulation of HLA class I, possibly explaining the positive prognostic effect of patients with loss of HLA class I expression. However, NK cell infiltration at the tumor site is scarce, indicating that tumor staining for NK cells might be minimally informative (40).

In conclusion we were able to identify local immune escape mechanisms of colon cancer, where the presence of Foxp3+ cell infiltration favors a better prognosis, indicating CTL activity. HLA-E and -G expression might play a pivotal role in distant immune escape mechanisms, where in case of loss or downregulation of HLA class I, HLA-E and -G expression determines distant metastases and prognosis of colon cancer patients. Furthermore we were able to determine three distinct survival patterns in colon cancer patients based on immune surveillance. In the future these findings might contribute to better treatment allocation and maybe even the development of new cancer immuno-therapies.

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CHAPTER 10

Summary, general discussion, conclusions and future directions

Colon cancer represents a major public health problem, accounting for more than 1 million new cases diagnosed each year and more than half a million deaths worldwide (1,2). While earlier diagnosis and advances in treatment have considerably improved survival in recent years, further progress is needed. Challenges include not only the development of new, less toxic adjuvant treatment regimens or discovering the optimal treatment sequence incorporating new molecular targeted therapeutic agents but more importantly, the optimal selection of patients for these adjuvant therapy strategies (3). The benefits of adjuvant chemotherapy have been most clearly demonstrated in patients with node-positive (stage III) disease, who have an approximately 30 percent reduction in the risk of disease recurrence and a 22 to 32 percent reduction in mortality with chemotherapy (4). Current guidelines therefore dictate adjuvant chemotherapy for all stage III colon cancer patients. The benefit of adjuvant chemotherapy in stage II disease remains controversial. A subset of patients within the stage II (node-negative) cohort are known to have a high risk of distant metastasis comparable to the stage III patient cohort and might therefore benefit from adjuvant treatment (5,6). Five large clinical trials have addressed this benefit in populations consisting either entirely or predominantly of stage II disease (7-11). Based upon this data, although most trials show a disease-free survival benefit of adjuvant therapy in stage II, the magnitude of this benefit is too small to treat all stage II patients with adjuvant therapy. The American Society of Clinical Oncology in 2004 therefore recommended a set of criteria, based upon the experience in stage III disease populations and data of the MOSAIC study, consisting of clinico-pathologic variables based on which high risk stage II patients can be identified (9,12). These criteria are: fewer than 12 sampled lymph nodes in the resection specimen, T4 lesions, tumor perforation at clinical presentation, poorly differentiated tumor histology and/or peri-neural, lymphatic or vascular invasion (12). Recent data from the Dutch Surgical Colorectal Audit (DSCA) points out that in the Netherlands only 18% of the stage II patients, indicated as high risk based on these recommendations, did actually receive adjuvant treatment (13). This implies that although the recommendations are part of the national guideline, they are not considered standard of care by the involved clinicians. This might be due to the circumstantial evidence the recommendations are based on. What are needed to better assist the clinicians in this process of treatment allocation are better diagnostic tools for the assessment of clinical prognosis and therapy response. Molecular tumor-derived biomarkers can serve as such prognostic and predictive biomarkers. Introduction of these biomarkers, together with the TNM criteria and possibly the ASCO criteria, might provide clinicians with a more feasible and evidence-based staging system.

In this thesis we applied a tumor biology-directed approach focusing on biological determinants of the tumor's growth and metastatic potential in order to identify such molecular biomarkers. The focus was on prognostic biomarkers, and mainly for stage II and III colon cancer patients. In the first part of the thesis we focused on biomarkers of apoptosis and proliferation. In the second part the focus was on studies on tumor-immune interactions.

PART 1 BIOMARKERS OF APOPTOSIS AND PROLIFERATION

Apoptosis or programmed cell death is an intriguing process regulated at multiple levels. Based on the stimulus presented, two pathways initiating the apoptotic process can be identified; an extrinsic pathway typically activated in immune responses and an intrinsic pathway activated for example by stimuli reflecting DNA damage (14). Both pathways converge at the level of the caspase cascade eventually causing proteolytic activation of the executioner caspase-3 (15). This protein acts as a cellular disassembly machine responsible for the morphological features that hallmark apoptotic cell death.

We have previously shown that the level of apoptosis in rectal cancer resection specimens was directly related to the development of local recurrence in clinical follow-up (16,17). To determine whether the pathway of apoptosis also harbors clinical prognostic relevance in colon cancer patients we executed an extensive review of the literature searching for prognostic biomarkers related to the apoptotic pathway in colorectal cancer (**Chapter 2**). We identified 26 potential biomarkers, studied with immunohistochemistry (IHC) in primary colorectal cancer patients of which expression data was related to patient outcome. The data, however, showed none of these single biomarkers to be of independent prognostic relevance or appropriately represented outcome of the apoptotic pathway. Under normal circumstances both apoptosis and proliferation play a pivotal role in the maintenance of tissue homeostasis (18). Disruption of either one of these processes contributes to the hallmarks of cancer: biological capabilities a cell acquires during the process of tumorigenesis (19,20). Our review pointed out that multi-marker studies describing combined analysis of several biomarkers, related to both apoptosis and proliferation, showed the most promising results with respect to predicting patient outcome (21,22).

Previously, Michael-Robinson *et al.* reported on prognostic value of an AI:PI (apoptotic index: proliferation index) ratio in a small cohort of colorectal cancer patients (23). Prompted by these results and the conclusions of our review of the literature, we proceeded with the study described in **Chapter 3**. In this chapter combined markers of tumor levels of cell proliferation and apoptosis were tested for their prognostic relevance in a large cohort of colon cancer patients using IHC on a TMA (Tissue Micro Array). This TMA technique combined with IHC enabled us to perform a rapid, cost-effective and high-throughput analysis of several molecular markers at protein level (24). The Ki67 antibody was used to determine level of tumor cell proliferation and we determined the tumor apoptotic levels based on the tumor cell IHC expression levels of activated (cleaved) caspase-3. Although many studies, including those by Michael-Robinson *et al.*, have used M30 IHC to determine tumor apoptotic levels, we choose to use IHC-determined expression of activated caspase-3 (23). First of all, because of our good experience with biochemical assays determining activated level of caspase-3 in previous studies (16,17,25). And secondly, studies have shown a superiority of anti-activated caspase-3 antibodies in determining the level of tumor apoptosis compared to M30 antibodies (26). Our

study revealed that combined analysis of both Ki67 and activated caspase-3-based levels of tumor proliferation and apoptosis into one CAP parameter (Combined Apoptosis Proliferation parameter) was significantly related to clinical outcome in a large cohort stage I-IV colon cancer patients. The direction of this correlation depended on both tumor location, and indirectly tumor microsatellite status, and TNM stage.

Since we now constituted the prognostic qualities of this combined parameter we further focused on the clinical value of the combined analysis of tumor apoptotic and proliferation levels in colon cancer patients in Chapter 4&5. In these chapters we studied both apoptosis and proliferation using biochemical assays. The advantages of using biochemical assays in the clinical setting over immunohistochemistry are the highly accurate performance of these assays on only limited amount of fresh frozen tissue that can be easily acquired by a biopsy or during surgery. Furthermore, the results are less influenced by inter-observer variety (27). More importantly, biochemical assays provide information on a functional level of the enzymatic activities of a protein as opposed to providing solely expression levels when using IHC. As mentioned before we already had good experience with analyzing the tumor apoptotic level with biochemical assays determining the activated level of caspase-3 (16,25). In **Chapter 4** the use of the level of cyclin-dependent kinase 1 specific activity (CDK1 SA), determined with a biochemical assay, as a predictor of tumor recurrence and patient outcome was studied. It is hypothesized that increased levels of intra- tumoral CDK1 SA reflects higher tumor proliferation rate. The expression of this kinase is constitutive in cells, but the enzymatic activity changes according to the cell cycle phase, and thus associates with cell proliferation. Previously the proliferation rate of tumor cells has been studied with many different methods. Chapter 3 for example demonstrates the use of ki67 IHC, but also ³H-thymidine/BrdU incorporation and PCNA IHC have been widely used methods. None were found to be useful in a clinical setting (28,29). The prognostic value of the biochemical assay determining CDK1 SA had already been proven in two large, independent cohorts of breast cancer patients (30,31). Therefore we choose to determine the prognostic qualities of this particular assay in colon cancer patients. In the study described in chapter 4, that included patient cohorts from both the Netherlands and Germany, the prognostic quality of CDK1 SA was studied in stage II colon cancer patients. Furthermore, the prognostic value of CDK1 SA was compared to another frequently studied prognostic biomarkers in stage II colon cancer: the BRAF V600E mutation status and the high-risk identification criteria recommended by ASCO (12,32). CDK1 SA was proven to be an independent prognostic predictor of distant tumor recurrence. Interestingly, high CDK1 SA was significantly related to a microsatellite stable tumor phenotype. This is probably due to the fact that the regulation of CDK1 activity is orchestrated at cellular checkpoints. The patient's tumor microsatellite status might induce changes in the structure and function of proteins related to these checkpoints, especially if microsatellites are present in key genes in these processes, eventually affecting the level CDK1 activity and tumor cell proliferation. When CDK1 SA is studied as a biomarker

for clinical application the patient's tumor microsatellite status should therefore always be taken into account.

In **Chapter 5** we validated the prognostic quality of the combined analysis of proliferation and apoptosis when both determined with biochemical assays, CKD1SA reflecting tumor cell proliferation and caspase-3 activity reflecting tumor cell apoptosis, in a cohort of stage II colon cancer patients taking into account the patients tumor microsatellite status. In this cohort, low levels of tumor cell CKD1SA and high levels of caspase-3 activity were independent predictors of better DFS (CKD1SA HR 9.6, p-value 0.029 and caspase-3 activity HR 0.5, p-value 0.03). The combined results of these assays, correctly classified 87.5% of the stage II MSS patient as high risk of developing distant metastasis.

In **summary**, we demonstrated the clinical importance of combined analysis of the level of tumor cell apoptosis and proliferation in chapter 3 and the use of a biochemical assay reflecting tumor proliferation and apoptosis in chapter 4. Furthermore, in chapter 5 the results of our combined analysis were compared to a subset of the ASCO criteria, and showed to perform equally with respect to test specificity and sensitivity (12). Thus, we were able to demonstrate that the use of our apoptosis/proliferation parameter, and taking into account the tumor microsatellite status, is of additive value in the high-risk patient identification.

PART 2 TUMOR-IMMUNE INTERACTIONS

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The immune system has been attributed an important role in controlling tumor growth and metastasis (33–36). New insight emerging from the growing field of cancer research have led to the addition of two hallmarks, gained during tumor development, to the six biological capabilities tumor cells have acquired in the multistep process of malignant transformation as originally described by Hanahan and Weinberg in 2000 (20,37). One of these two emerging hallmarks is the capability of tumor cells to evade immune destruction. It has become clear that tumor cells during their transformation, and in interaction with their host, create a so-called tumor micro-environment (38). One of the characteristics of this tumor micro-environment is interaction of tumor cells with the immune system. The tumor immune surveillance hypothesis which postulates that the immune system can identify cancerous cells and eliminate them, probably usually before they can cause harm is based on this observed interaction (39). Based on this hypothesis studying tumor immune-interactions provides us not only with new biomarkers as described in chapter 8 and 9, it also provides a possibility to develop new treatment strategies for the high-risk patient cohort.

One of the new immune-based treatment strategies, the use of vaccines, exploits activation of the patient's immune system as a therapeutic modality. In **Chapter 6** we provide a review of the literature on clinical trials in which both tumor cell-derived vaccines as well tumor antigen-derived vaccines are deployed. To eliminate tumor cells a vaccine needs to induce a robust tumor-specific immune

response. Furthermore, the response should be uniquely target tumor cells. Based on the literature reviewed we therefore listed some key features required to obtain clinical results in colon cancer vaccine strategies. Preferably, a vaccine should target tumor-specific antigens, enhance the number of effector T cells but not the number of suppressive T cells, and create a T-cell friendly and supportive tumor micro-environment. Previously we established the safety and immunogenicity of a p53 synthetic long peptide (p53-SLP®) vaccine (40). This vaccine is comprised of mutated, and therefore functionally not activated, P53 that is overexpressed in 34-45% of the colon cancer patients while wild type p53 is expressed at low levels (41). Results of this study showed we were able to induce a p53- specific CD4⁺ T cell response. However, though the vaccine seemed to induce Type 1 T-helpers (Th1) cells, it resulted in only low amounts of key cytokines such as interferon- γ (IFN- γ) and IL-2. It was concluded that a p53 -specific Th-response was induced, but probably not properly polarized. To optimize our vaccine we compared the design of our p53 synthetic long peptides (p53-SLP®) vaccine to the vaccine key features we described in chapter 6. We concluded that in order to polarize the Th-response properly and actually benefit from tumor-specific Th-cells at the tumor site, an immune stimulating treatment modality should be added to vaccine.

In **Chapter 7** we investigated if the addition of such an immune-stimulating treatment modality, interferon- α (IFN- α) in addition to the p53-SLP® vaccine, is safe and would actually result in higher production of key cytokines such as IFN- γ representing a more Th1-polarized response and possibly resulting in the presence of more tumor-specific CD4⁺ T cells at the tumor site. In this phase I/II clinical trial 11 colorectal cancer patients were enrolled after successfully being treated for metastatic disease. The results showed that besides from being safe, the toxicity was limited to Grade 1 or 2 with only small ongoing swellings at the vaccination site. All patients harbored p53- specific T-cells after vaccination and most patients showed p53-specific antibodies. More importantly, compared to the previous trial, with the addition of IFN- α the frequency of p53-specific T cells producing IFN- γ improved significantly. These data are very promising, however, the clinical introduction of this type of vaccination is still a long way ahead. Not only are the minimal requirements of a vaccine-induced immune responses to obtain a clinical tumor response at this moment practically undefined, the responses we were able to induce by are all means too small.

In chapter 8 and 9 we focused on studying the tumor micro-environment and tumor immune- interactions in order to identify immune-related biomarkers. These biomarkers might not only aid us in the identification of high-risk stage II patients, they might also be used in the future to select patients for vaccine-based adjuvant therapies. For this purpose in **Chapter 8** we studied the presence in tumors of Foxp3⁺ regulatory T-cells and of CD8⁺ cytotoxic T-cells (CTLs) in relation to tumor cell HLA class I expression in colon cancer patients and we related the data obtained to patient outcome parameters. The adaptive immune system and its key players, CTLs, play an important role in controlling tumor growth and metastasis (36). Tumor cells may develop a phenotype that results from downregulation or

loss of one or more HLA class I alleles, that makes them less sensitive to immune surveillance by escaping CTL recognition (34,35,42,43). Another mechanism to evade immune surveillance is the attraction of immunosuppressive regulatory T cells into the tumor micro-environment. Both features have been shown to be of clinical relevance in various types of solid tumors (44–47). In our study neither the presence of Foxp3+ cells nor the presence of CD8+ cells was statistically significant related to patient outcome. A ratio, calculated based on the presence of these cells, however, significantly predicted clinical outcome. Relatively high numbers of CD8+ cells compared to the number of Foxp3+ cells was related to better distant recurrence free survival suggesting a mutual functional relationship (figure 4b, chapter 8). Furthermore, we also included data on the tumor HLA class I status and the tumor microsatellite status. Although we were not able to demonstrate the precise effects of the HLA class I status and tumor microsatellite status on patient outcome, the data did underline the need of a comprehensive approach when studying the effect of biomarkers representing the interplay between a tumor and the multi-faceted immune system in predicting the patient outcome.

The goal of the study presented in **Chapter 9** was to establish distinct patterns that reflect mechanisms involved in ‘tumor immune escape’ and relate these to patient outcome using a comprehensive approach based on the conclusion of the study described in chapter 8. Because of the advantages listed previously again a TMA of stage I-IV colon cancer patients the appropriate tool to study these patterns was. Markers included were presence of Foxp3+ cells, tumor expression of HLA class I (HLA-A, -B, and -C) and the expression of non-classical HLA-E and -G. The quantitative data of these markers was combined into three immune-phenotypes; a high, intermediate, and low immune susceptible phenotype. After careful analysis the three profiles were found to represent significant, independent clinical prognostic profiles. Although we are aware of the limitations of this type of retrospective studies and the need for further evaluation and validation larger cohorts, we found the results to be very promising.

CONCLUSIONS AND FUTURE DIRECTIONS

This thesis focuses on finding biomarkers that reflect biological hallmarks of tumorigenesis in order to develop tools that might aid in the clinical identification of high-risk stage II colon cancer. We found that based on biochemical assays the combined analysis of apoptosis and proliferation might guide this identification, predominantly in those patients with microsatellite stable tumors. When studying mechanisms involved in tumor-immune interaction it was also the combined analyses of key-biomarkers that harbored clinical potential. These biomarker-based phenotypes might also aid in the future development of tumor vaccine-based therapeutical strategies for colon cancer as they might be used for patient selection that are expected to benefit from such therapeutical approach.

Although the results are promising, introduction of any of the biomarker profiles studied in this thesis are not yet ready for clinical introduction. As described in chapter 2 of this thesis Pepe *et al.* proposed a five step program which can serve as a guideline for the clinical introduction of a biomarker (48). At the moment the status of our biomarkers does not exceed the steps 1-2; the pre-clinical exploratory phase and clinical assay and validation phase. There are various issues that need to be addressed before any of these marker profiles will make it 'from bench to bedside'. For example, the role of the tumor microsatellite status needs to be addressed more thoroughly. Previous studies have shown that the tumor microsatellite is related to patient outcome, while others have suggested this is not due to the microsatellite status but is related to tumor location and its embryonic tissue origin or the tumor's mutational load (49-52). Therefore, this point needs much more attention and research.

Altogether the results of the studies presented in this thesis emphasize the importance of studying the tumor biological context in order to develop prognostic biomarkers of clinical significance. Tumor growth and -progression is the result of a multi-step and multi-faceted process. The strength of our biology-based approach lies in the fact that we studied key-elements that reflect outcome of processes such as the regulation tumor cell apoptosis, proliferation and interaction with the immune system, all within the tumor micro-environment. Through combining results we were able to create 'tumor phenotypes' that reflect tumor aggressiveness. These combined biomarker-based phenotypes much better reflect tumor biology and thus clinical outcome than any approach based single marker studies, as most are in scientific literature. To warrant the clinical introduction of biomarker-based phenotypes in the near future and in order to develop a tumor phenotype that will ultimately reflect the status of all hallmarks of cancer, these studies must be further extended. Based on this thesis we are able to state with confidence that biomarker-based phenotypes have the potential to accurately determine the clinical behavior of individual tumors and therefore a patient's individual clinical prognosis and thus may provide a solid guide for treatment decisions.

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CHAPTER 11

Nederlandse samenvatting

De afgelopen jaren is er veel aandacht besteed aan de vroegtijdige opsporing en behandeling van het coloncarcinoom. En niet onterecht. Dikkedarmkanker heeft een belangrijke impact op de volksgezondheid met wereldwijd jaarlijks ruim 1 miljoen nieuwe patiënten bij wie de ziekte wordt vastgesteld (1, 2). Daarbij overlijden er per jaar nog altijd meer dan een half miljoen mensen aan de gevolgen van deze ziekte. De oorzaak van overlijden is vaak het gevolg van uitzaaiingen op afstand (metastasen), zoals naar de lever (1).

Dankzij ontwikkelingen in de wetenschap, die hebben geleid tot o.a. eerdere diagnose en betere behandeling, is de overleving van mensen, bij wie deze ziekte de afgelopen 10 jaar werd vastgesteld, toegenomen. Desondanks valt er nog veel winst te behalen. De basis van de behandeling van het coloncarcinoom blijft chirurgische resectie van de primaire tumor. Echter, sinds het begin van de jaren '90 is het duidelijk geworden dat, wanneer er sprake is van uitzaaiing naar lokale lymfeklieren, er na resectie een belangrijke rol is weggelegd voor nabehandeling met chemotherapie. Het is op dit moment standaardprotocol om alle stadium III patiënten deze adjuvante therapie aan te bieden. In tabel 1 wordt weergegeven hoe deze indeling in stadia voor dikkedarmtumoren werkt. Deze nabehandeling, ook wel adjuvante chemotherapie genoemd, heeft geleid tot een daling van het risico op terugkeren van de ziekte: voor metastasering op afstand in deze patiëntengroep tot 30%, en een verlaging van het overlijdensrisico naar 32% (3).

Bij stadium II patiënten is nabehandeling met chemotherapie controversieel. Voor een kleine groep stadium II patiënten is bewezen dat zij een grote kans hebben op het ontwikkelen van metastasen op afstand (met name naar de lever) wat natuurlijk zeer nadelige gevolgen heeft voor de overleving van deze patiënten. Deze patiënten zouden dus profijt kunnen hebben van een standaard nabehandeling met chemotherapie (4), ware het niet dat deze behandeling gepaard gaat met een hoge mate van morbiditeit. De algemene opvatting is dan ook dat het standaard nabehandelen van alle stadium II patiënten, waarvan een groot deel zonder behandeling nooit uitzaaiing op afstand zou ontwikkelen, niet opweegt tegen de risico's van deze, in de meeste gevallen dus onnodige, behandeling (5).

Vanwege de potentiële overlevingswinst in de selecte groep stadium II patiënten die uitzaaiingen ontwikkelt, is er de afgelopen jaren veel aandacht besteed aan het identificeren van deze patiënten. De American Society of Clinical Oncology (ASCO) heeft in 2004 een aanbeveling gedaan waarin een aantal criteria zijn opgesteld die artsen moet helpen deze hoogrisicogroep stadium II colonkankerpatiënten te identificeren (6). Het gaat dan om patiënten waarbij bij pathologisch onderzoek minder dan 12 lymfeklieren in het resectiepreparaat worden aangetroffen, patiënten waar bij de tumor reeds tot in de buitenwand van de darm (T4-tumoren genoemd) is gegroeid of waarbij deze al geperforeerd is door de darmwand heen bij diagnose, patiënten waarvan de tumor slecht gedifferentieerd is, of patiënten waarbij de tumor ingroei laat zien in de kleine bloed- en of lymfevaten of rondom de zenuwbanen bij pathologisch onderzoek. Uit onderzoek binnen een database, waarin vrijwel alle patiënten zijn geregistreerd, die in 2010 in Nederland aan een coloncarcinoom werden geopereerd (uitgevoerd door de DSCA, de Dutch Surgical

Tabel 1

Indeling van dikkedarmtumoren naar ziektestadium op basis van de TNM 5 criteria uit 1997

Stadium	T	N	M
I	1-2	0	0
II	3-4	0	0
III	1-4	+	0
IV	1-4	+	+

Het TNM classificatiesysteem van maligne tumoren is ontwikkeld door de International Union Against Cancer (UICC) en wordt wereldwijd gebruikt voor de classificatie van dikkedarmtumoren. De T staat voor tumor en beschrijft de lokale uitbreiding. T1 betekent groei van de tumor tot in de submucosa, het slijmvlies van de darm, maar er niet doorheen. T2 staat voor tumorgroei tot in de spierlaag van de darm, de muscularis propria, maar er niet doorheen. De T3 codering wordt gebruikt als de tumor doorgroeit tot in de buitenlaag van de darm, de subserosa of in het omliggend vet en een tumor wordt als T4 gecodeerd als deze ingroeit in omgevende organen of door het buikvlies heen. De N staat voor mate van metastasering naar lymfeklieren. Een N0 codering betekent dat er geen sprake is van kliermetastasen, een N+ codering geeft aan dat dit wel het geval is. De M staat voor metastase op afstand. De code M0 betekent dat er geen sprake is van metastasering op afstand, M+ betekent dat de tumor of afstand gemetastaseerd is bijvoorbeeld naar de lever of long.

Colorectal Audit), blijkt dat 37% van de colonkankerpatiënten werden geopereerd in verband met een stadium II tumor (7). Van deze patiënten werd 13% op basis van de ASCO-criteria ingeschat als hoog risico. Opvallend genoeg werd slechts 18% van deze hoogrisicopatiënten daadwerkelijk nabehandeld met chemotherapie ondanks dat de criteria van de ASCO op dat moment al als aanbeveling waren opgenomen in de landelijke richtlijn 'behandeling van dikkedarmkanker' welke is na te lezen op www.oncoline.nl. We kunnen dus concluderen dat de nabehandeling met chemotherapie van hoogrisico stadium II patiënten in Nederland nog niet tot de standaard hoort. Een reden hiervoor zou kunnen zijn dat een groot deel van de behandelend artsen (o.a. oncologen en chirurgen) de classificatie van hoogrisicopatiënten op basis van de ASCO-criteria onvoldoende vindt om over te gaan tot een dergelijke invaliderende nabehandeling. Kortom, de behandelaars van patiënten met een coloncarcinoom in Nederland, maar ook in de rest van de wereld zijn op zoek naar een betrouwbare methode om de hoogrisico stadium II patiënten te kunnen identificeren.

De afgelopen jaren is er daarom wereldwijd veel onderzoek gedaan naar betere identificatie van deze hoogrisico stadium II patiënten. Er is met name veel winst behaald door studies naar het gebruik van biomarkers. Biomarkers zijn organische substanties zoals bijvoorbeeld cellen, eiwitten en genen, die in het bloed of het tumorweefsel van patiënten in het laboratorium kunnen worden geanalyseerd. Door de aan- of juist afwezigheid van deze biomarkers te analyseren, bijvoorbeeld in relatie tot de overleving van patiënten, kan worden bestudeerd of deze voorspellend zijn voor de prognose van patiënten.

Ook in dit proefschrift ligt de focus op het gebruik en de ontwikkeling van biomarkers die informatie kunnen geven over de prognose van een patiënt met darmkanker. In deel 1 hebben wij gekeken naar een aantal processen die van belang

zijn voor tumorgroei en -ontwikkeling, zoals geregleerde celdood (apoptose) en ongecontroleerde celdeling (proliferatie). In deel 2 hebben wij de interactie tussen tumoren en het afweersysteem bestudeerd.

DEEL 1 BIOMARKERS GERELATEERD AAN APOPTOSE EN PROLIFERATIE

Vernieuwing van cellen door celdeling is een proces dat continu plaatsvindt in vrijwel alle weefsels in het lichaam, maar met name in weefsels met veel slijmvlies zoals de darm. Om te voorkomen dat dit resulteert in woekering van cellen en dus tumorvorming worden oude en/of beschadigde cellen regelmatig afgebroken. Dit resulteert in een balans van aanmaak en afbraak van cellen die ook wel weefselhomeostase wordt genoemd (8-10). Apoptose, ook wel geregleerde celdood genaamd, draagt in belangrijke mate bij aan de afbraak van deze oude en beschadigde cellen (11). Het is een complex proces, dat op meerdere niveaus wordt geregleerd. Het proces kan door de cel zelf worden geïnitieerd; van binnenuit (intrinsiek), bijvoorbeeld naar aanleiding van DNA-schade, of door stimuli van buiten af (extrinsiek), bijvoorbeeld door activering van het immuunsysteem (11). Beide routes leiden tot de activering van een bijzondere groep eiwitten, de caspases (12), die op basis van hun enzymatische activiteit in staat zijn een cel af te breken.

Eerder onderzoek uit ons laboratorium heeft aangetoond dat de hoeveelheid aan apoptotische activiteit in het tumorweefsel van patiënten met een endeldarmtumor significant gerelateerd is aan de mate van lokale terugkeer van tumor in deze patiëntenpopulatie (13, 14). In de literatuur is deze relatie (tussen de mate van tumorcelapoptose en de prognose van patiënten) ook vastgesteld voor dikkedarmtumoren (15). Bepaalde eiwitten die onderdeel zijn van het proces leidend tot apoptose in dikkedarmtumoren zouden daarom kunnen functioneren als biomarkers. In **Hoofdstuk 2** geven we een overzicht van de wetenschappelijk literatuur die op dit moment beschikbaar is op het gebied van biomarkers gerelateerd aan apoptose. Hierbij hebben wij met name gekeken naar prognostische relevantie en specifiek ook naar studies waarin deze biomarkers met behulp van immunohistochemie werden bepaald. Immunohistochemie (IHC) is een techniek waarbij de aanwezigheid van een bepaald eiwit kan worden aangetoond door deze te labelen met antilichamen die specifiek binden aan dit eiwit. In de literatuur hebben we 26 potentiële biomarkers, gerelateerd aan apoptose en bestudeerd met IHC, gevonden. De functie van deze biomarkers binnen het proces van apoptose en de resultaten van de afzonderlijke studies worden in het overzichtsartikel in hoofdstuk 2 beschreven. De belangrijkste conclusie van dit artikel was dat geen enkele biomarker op zich zelf, dus als 'single' biomarker, significant gerelateerd was aan de klinische prognose van patiënten. Derhalve zijn deze biomarkers op dit moment als 'single' biomarkers niet bruikbaar in de kliniek voor de identificatie van hoogrisico stadium II patiënten.

Een verklaring voor het uitblijven van prognostische relevantie van deze zogenaamde 'single biomarkers' kan zijn dat voor het voorkomen van tumorvorming niet alleen een intact proces van apoptose nodig is, maar ook een goed gereguleerde nieuwvorming of proliferatiecyclus. Immers beide dragen bij aan weefsel-homeostase. Ons literatuuronderzoek toonde dat een combinatie van biomarkers, zowel gerelateerd aan apoptose als aan proliferatie, betere resultaten geeft voor wat betreft het voorspellen van de prognose van patiënten. Vanwege deze betere voorspellende waarde van de combinatie van biomarkers zowel betrokken bij apoptose als bij proliferatie hebben wij een studie opgezet welke wordt beschreven in **Hoofdstuk 3**. Hierin kijken we naar een combinatie van biomarkers, ki67 en geactiveerd caspase-3, die respectievelijk betrokken zijn bij celproliferatie en apoptose. Wij bepaalden deze biomarkers op een zogenaamde 'tissue micro array' (TMA) met de eerder genoemde immunohistochemie (IHC) techniek. Het voordeel van het gebruik van een dergelijke TMA is dat de analyse goedkoper is en met een minimaal gebruik van weefsel kan worden uitgevoerd. De combinatie van de ki67 en caspase-3 markers werd geanalyseerd op tumorweefselsamples van bijna alle patiënten die in een periode van 10 jaar (tussen 1990-2000) in het LUMC werden geopereerd aan een coloncarcinoom. De resultaten van dit onderzoek toonden aan dat de gecombineerde aanwezigheid van deze markers, verwerkt in de door ons ontwikkelde CAP (Combined Apoptosis and Proliferation) parameter, zowel correleerde met prognose, de locatie van de tumor in de dikke darm, als met tumorstadium.

Ondanks de positieve uitkomst van de studie in Hoofdstuk 3 is een dergelijke uitgebreide vorm van IHC voor toepassing van deze combinatie van biomarkers in de kliniek niet haalbaar. Alhoewel IHC relatief goedkoop is kost een dergelijke gecombineerde analyse veel tijd (16). Maar een belangrijker nadeel is dat de analyse nog niet objectief kan worden uitgevoerd. Alle afzonderlijke bepalingen worden door analisten op het oog gescoord hetgeen kan leiden tot variaties tussen de verschillende beoordelaars; interbeoordelaarsvariatie genoemd (16). Op dit moment wordt in ons laboratorium gewerkt aan een geautomatiseerde methode waarbij de analyse van de IHC resultaten door een computer wordt uitgevoerd. Deze automatische analysemethode is echter nog niet gevalideerd en voor toepassing op individuele patiënten relatief kostbaar. Om de klinische toepasbaarheid van onze parameter te realiseren werd daarom gekozen tot het ontwikkelen van een test waarbij zowel de mate van apoptose als proliferatie kunnen worden bepaald, op minimale hoeveelheid materiaal door middel van real-time testing. Het voordeel hiervan is dat een dergelijke analyse geen gebruik maakt van IHC en daardoor een gestandaardiseerde uitkomstmaat biedt die niet gevoelig is voor de eerder genoemde interbeoordelaarsvariatie. Voor het analyseren van de mate van apoptose is een dergelijke test al ontwikkeld en in het verleden met succes uitgevoerd in ons laboratorium (17). In deze test wordt naast de hoeveelheid van het aanwezige caspase-3 enzym ook de enzymatische activiteit van het eiwit bepaald. Wereldwijd wordt deze bepaling geaccepteerd als een marker voor de mate van apoptotische activiteit in tumorweefsel (16).

Een dergelijke marker, waarbij gebruik wordt gemaakt van enzymatische activiteit, voor tumorcelproliferatie was nog niet voorhanden voor het coloncarcinoom. In **Hoofdstuk 4** hebben wij daarom een nieuwe marker, ontwikkeld door het Japanse bedrijf Sysmex, die representatief is voor de hoeveelheid tumorcelproliferatie, getest voor klinische relevantie in samenwerking met collegae van de technische universiteit van de Rechts an der Isar Klinikum in München. Deze marker is genaamd CDK1SA ('cyclin dependent kinase 1 specific activity'). Het CDK1 eiwit is nauw betrokken bij de verschillende fases van celdeling, die in normale cellen worden doorlopen en functioneert als een poortwachter in de regulatie van dit proces(18). Een hoge mate van CDK1 activiteit is bewezen gerelateerd te zijn aan een hoge mate van celproliferatie en eerdere studies uit ons laboratorium hebben een klinische relevantie van deze marker met betrekking tot prognose in borstkankerpatiënten aangetoond (18-20). Ter analyse van de klinische relevantie van deze marker met betrekking tot de stadium II coloncarcinoompatiënten werd materiaal afkomstig van twee min of meer gelijkwaardige patiëntengroepen uit Duitsland en Nederland gebruikt. De resultaten van dit onderzoek toonden dat de specifieke enzymatische activiteit van CDK1 (CDK1SA), in essentie op dezelfde wijze bepaald als de enzymatische activiteit van caspase-3, significant gerelateerd is aan de kans op het ontwikkelen van een metastase op afstand. Opvallend genoeg was er een sterke relatie met de tumormicrosatellietstatus. Microsatellieten zijn kleine repeterende nucleotidensequenties in het DNA. Een toename in de frequentie van deze repeterende sequenties ontstaat door niet goed functionerend DNA herstelsysteem (het MMR, mismatch repair systeem) (21-23). Dit uit zich in instabiliteit van het genoom wat microsatelliet-instabiliteit (MSI) wordt genoemd. Onder normale omstandigheden zorgen de eiwitten die het MMR vormen er voor dat fouten, die tijdens celdeling spontaan kunnen ontstaan en resulteren in de vorming van microsatellieten, worden gecorrigeerd. In sommige tumoren kunnen MMR eiwitten hun functie niet goed uitvoeren doordat er of wel een mutatie optreedt in het gen verantwoordelijk voor de productie van deze MMR eiwitten, of doordat er te weinig MMR eiwitten worden geproduceerd door hypermethylering van de promotor van een van de betrokken genen, dit resulteert in MSI. Patiënten met tumoren waarvan het DNA MSI laat zien hebben over het algemeen een betere prognose (23). Wij concludeerden dan ook dat als CDK1SA wordt geanalyseerd ten einde een uitspraak te doen over de prognose van een patiënten, de tumormicrosatellietstatus ook altijd in overweging dient te worden genomen.

Aangezien we in hoofdstuk 4 hebben aangetoond een betrouwbare biomarker te hebben ontwikkeld, representatief voor de mate van tumorproliferatie, hebben wij in **Hoofdstuk 5** gekeken naar de combinatie van deze biomarker met de reeds bekende biomarker voor de mate van apoptose, het geactiveerd caspase-3. In een patiëntengroep bestaande uit alleen stadium II colonkankerpatiënten werden de resultaten van beide analyses gecombineerd. We waren in staat om in dit cohort met behulp van onze assay in 87.5% van de stadium II patiënten met microsatellietstabile tumoren correct te classificeren als hoogrisicopatiënten.

Kortom, de gecombineerde analyse van biomarkers die zowel betrekking hebben op de mate van apoptose in tumorweefsel als de mate van proliferatie, kan worden gebruikt om hoogrisico stadium II patiënten met colontumoren te identificeren. In hoofdstuk 5 werd onze classificatie, gebaseerd op gecombineerde biomarkers, tevens vergeleken met de classificatie in hoog- en laagrisicopatiënten op basis van de door de ASCO voorgestelde criteria (6). Vooralsnog was onze gecombineerde analyse even specifiek als sensitief in het identificeren van hoogrisicopatiënten als wanneer de ASCO criteria werden toegepast. Dit biedt perspectieven voor een eventuele, gecombineerde toepassing in de toekomst.

DEEL 2 INTERACTIE TUSSEN HET COLONCARCINOOM EN HET IMMUUNSISTEEM

Een belangrijke rol in de controle van tumorgroei en de ontwikkeling van metastasen is weggelegd voor het immuunsysteem(24-27). In 2000 werden door Hanahan en Weinberg een zestal kerneigenschappen van tumorcellen ook wel 'Hallmarks of cancer development' genaamd beschreven (28). Het betreft veranderingen die normale cellen moeten ondergaan om zich te ontwikkelen tot kwaadaardige kankercellen. Deze kenmerken hebben betrekking op het in stand houden van de proliferatie en onbeperkte celdeling, het vermijden van apoptose, het vermogen tot invasie van weefsel en het ontwikkelen van metastasen op afstand en het vermogen tot het aanzetten van bloedvatvorming (29). Voortschrijdend inzicht in het proces van tumorgroei en ontwikkeling leidde er in 2011 toe dat er een tweetal kenmerken werden toegevoegd aan deze kerneigenschappen. Een daarvan was het vermogen van tumorcellen om vernietiging door het immuunsysteem te voorkomen. Tijdens het proces van maligne celtransformatie vindt er een belangrijke interactie plaats tussen de tumorcellen en het omringende weefsel, ook wel genaamd de tumor-microenvironment genoemd (29). In een belangrijke hypothese genaamd de 'tumor immune surveillance hypothesis' wordt gesteld dat het immuunsysteem van elk mens in staat is kankercellen te identificeren en te elimineren, nog voordat deze tot een kwaadaardigheid kunnen leiden (30). Deze hypothese vormde de basis voor de studies die worden gepresenteerd in het tweede deel van het proefschrift. In hoofdstuk 6 en 7 behandelen wij hoe het immuunsysteem van de patiënt gebruikt zou kunnen worden in de behandeling van kanker met behulp van zogenaamde tumorvaccins. In hoofdstuk 8 en 9 tonen wij vervolgens hoe deze interactie tussen de tumorcel en immuunsysteem ook kan worden gebruikt voor de ontwikkeling van nieuwe biomarkers.

Tumorvaccinatie

Onderzoek naar tumorvaccinaties is een veelbelovende stap in de richting van de ontwikkeling van nieuwe behandelstrategieën van het coloncarioom. Eind verleden jaar nog werd immunotherapie als behandeling van kanker verkozen tot de 'Breakthrough' van het jaar 2013 door Science Magazine. In het kort wordt bij dit

soort vaccinaties gebruik gemaakt van tumorspecifieke eiwitten (genaamd antigenen) om het immuunsysteem te activeren en zo de tumorcellen te vernietigen. In **Hoofdstuk 6** wordt een overzicht gegeven van de literatuur met betrekking tot een aantal klinische studies waarin dergelijke vaccins zijn onderzocht. Uit dit overzicht blijkt dat er een aantal kerneigenschappen zijn waar een tumorvaccin aan moet voldoen. Het vaccin moet veilig zijn in gebruik en, zoals al genoemd, gericht zijn tegen tumorspecifieke eiwitten. Het vaccin moet een reactie van specifieke T-cellen opwekken welke in staat zijn de tumorcellen te vernietigen en het moet een dusdanig effect hebben op de tumor-microenvironment dat deze T-cellen hun werk kunnen doen.

Binnen het LUMC, in een samenwerkingsverband tussen de afdeling Klinische Oncologie en de afdeling Heelkunde, is de afgelopen jaren gewerkt aan de ontwikkeling van het zogenoemde p53-SLP[®] vaccin, een synthetisch gefabriceerd p53 'long peptide' vaccin (31). Het eiwit p53 leent zich goed als doelwit voor tumorvaccinatie omdat het in bijna de helft van de colontumoren in grote hoeveelheden wordt geproduceerd terwijl de normale variant (het wildtype) van het eiwit in tumoren nauwelijks voorkomt (32). Eerdere studies hebben aangetoond dat het vaccin voldoet aan kerneigenschappen van een vaccin: het is veilig in gebruik en in staat om een specifieke (CD4+) T-celreactie op te wekken (31). Deze CD4+ T-celrespons is cruciaal voor de werking van een vaccin. De CD4+ T-cellen zorgen er namelijk voor dat CD8+ T-cellen, ook wel cytotoxische T cellen genaamd, kunnen ontstaan die gericht zijn tegen tumorcellen. Daarnaast ondersteunen ze de CD8+ T-cellen in hun functie: het herkennen van de tumorcellen op basis van het antigen wat verwerkt is in het vaccin en destructie van deze tumorcellen. Daarin spelen de cytokines interferon- γ (IFN- γ) en interleukine-2 (IL-2), geproduceerd door de CD4+ T-cellen, een belangrijke rol.

Helaas lieten de resultaten van de eerste studies met het p53-SLP[®] vaccin zien dat de opgewekte CD4+ T-celrespons, ook wel een Type 1 T-helperrespons genaamd, slechts leidde tot de productie van een zeer kleine hoeveelheid van deze belangrijke cytokines. De opgewekte respons was dus niet effectief genoeg. Daarom is getracht, kijkend naar de kerneigenschappen uit het literatuuronderzoek van hoofdstuk 6, het p53-SLP[®] vaccin te optimaliseren. De belangrijkste aanpassing richtte zich op het verbeteren van de tumor-microenvironment om zo de ontwikkeling van de CD4+ T-celrespons te stimuleren. Het nieuwe vaccin bevat dan ook naast het originele p53-SLP[®] vaccin, interferon-alpha (INF- α). Van deze stof is eerder beschreven dat het een positieve uitwerking heeft op het tumor-microenvironment (33).

In **Hoofdstuk 7** wordt de studie beschreven waarin het verbeterde vaccin werd getest op allereerst veiligheid maar natuurlijk ook op de mate waarin het vaccin in staat was een CD4+ T-celrespons te induceren. In de studie werd het verbeterde vaccin getest in 11 patiënten, allemaal succesvol behandeld voor gemetastaseerd coloncarcinoom. De resultaten lieten zien dat het verbeterde vaccin veilig was in gebruik; de enige bijwerkingen die werden gerapporteerd betroffen kleine zwelling van de huid rondom het geïnjecteerde gebied. Daarnaast blijkt uit de data van

de immunologische deelstudies dat alle patiënten een specifieke T-celrespons ontwikkelden tegen het vaccin. Maar misschien nog belangrijker, in vergelijking tot het p53-SLP® vaccin alleen leverde de combinatie van het vaccin met IFN- α significant meer IFN- γ p53-specifieke T-cellen op. Kortom, door toevoeging van IFN- α aan het bestaande vaccin werd het immunologisch resultaat duidelijk verbeterd. Deze data scheppen ruimte en mogelijkheden voor verdere ontwikkeling van het vaccin. Er zal met name nog veel aandacht moeten worden besteed aan het optimaliseren van het immunologisch resultaat. Het is namelijk nog niet bewezen dat de mate waarin wij nu een reactie hebben kunnen induceren ook daadwerkelijk een klinisch significant resultaat oplevert.

Biomarkers en de interactie tussen colontumoren en het immuunsysteem

In hoofdstuk 8 en 9 wordt, zoals eerder genoemd, aandacht geschonken aan de tumor, zijn omgeving op celniveau en de interactie die hier plaatsvindt tussen tumorcellen en het immuunsysteem. Het doel; het identificeren van biomarkers gerelateerd aan deze interactie welke van prognostische waarde zijn. In dit proefschrift wordt met name gefocussed op biomarkers van invloed op de identificatie van de hoogrisicopopulatie binnen het cohort van de stadium II colonkankerpatiënten. Het verworven immuunsysteem, ook wel het adaptieve immuunsysteem genaamd, bestaat uit meerdere typen cellen. De belangrijkste, met betrekking tot tumorgroei en -ontwikkeling, zijn de al eerder genoemde CD8⁺ cytotoxische T-cellen (CTLs) die tumorcellen kunnen herkennen en afbreken (25). Tijdens de maligne transformatie kan het uiterlijk (het fenotype) van een cel dusdanig gewijzigd worden dat de tumorcel feitelijk onherkenbaar wordt voor het immuunsysteem. Dit gebeurt door veranderde expressie van bepaalde eiwitten, humaan leucocytenantigen (HLA) genaamd, die voor een belangrijk deel de interactie met het immuunsysteem reguleren (26, 27). Van het HLA bestaan verschillende groepen of klassen bestaan. HLA klasse type I zorgt voor de interactie met de CTLs. Door verminderde expressie van de HLA klasse type I eiwitten zorgen tumorcellen er dus in feite voor dat ze onherkenbaar worden voor CTLs en zo ontsnappen aan destructie door het immuunsysteem, ook wel 'immune surveillance' genaamd (34, 35). Een andere manier om aan immune surveillance te ontkomen is onderdrukking van de interactie tussen tumorcellen en het immuunsysteem. Cellen die deze uitwerking hebben op het immuunsysteem zijn regulatoire T cellen, ook wel 'regulatory T cells' of Foxp3⁺ T-cellen genaamd (36-38). Door het aantrekken van deze cellen in de omgeving van de tumor wordt de aanval van het immuunsysteem op de tumor als het ware onderdrukt. In **Hoofdstuk 8** wordt bestudeerd of de aanwezigheid van CTLs, regulatoire T-cellen en de mate waarin het fenotype van een tumor op HLA klasse I niveau is veranderd, van invloed is op de prognose van colonkankerpatiënten. Omdat de CTLs en regulatoire T-cellen elkaar feitelijk tegenwerken hebben we ook gekeken naar de ratio tussen de aanwezige cellen van beide groepen. En inderdaad, relatief meer aanwezigheid CD8⁺ T-cellen dan regulatoire T-cellen had een positief effect op de prognose van deze patiënten met betrekking tot de duur van overleving zonder afstandsmetastasen. Ook in deze

studie werden meerdere factoren, zoals MSI-status, mogelijk van invloed op de prognose van patiënten meegenomen in de analyse. De belangrijkste conclusie van deze studie was dat de interactie tussen het immuunsysteem, de tumor en de tumoromgeving uit zoveel facetten bestaat dat ook op dit gebied meerdere biomarkers nodig zijn om het effect van de uitkomst van deze interactie op de overleving van patiënten te beschrijven.

In de studie beschreven in **Hoofdstuk 9** proberen we deze ingewikkelde interactie tussen tumorcel en immuunsysteem vast te leggen in aantal patronen waarin de verschillende markers werden gecombineerd en deze te relateren aan de klinische prognose van de patiënt. Er werd gekeken naar HLA-expressie, de aan- of afwezigheid van Foxp3⁺ cellen en de tumormicrosatellietstabiliteitstatus. Daarbij werd uitgebreider dan in hoofdstuk 8 gekeken naar het HLA-fenotype met meerdere markers zoals de klassieke HLA type I markers, HLA-A,-B en -C, maar ook niet-klassieke markers als HLA-E en -G. Op basis van de kwantitatieve IHC data van deze markers konden we 3 patronen onderscheiden. Een zogenaamd hoog, gemiddeld of laag immuunreactief tumorpatroon welke significant gerelateerd waren aan de prognose van patiënten. In de toekomst zal moeten worden gevalideerd in onafhankelijke patiëntenpopulaties of de door ons geïdentificeerde patronen inderdaad van klinisch prognostische waarde zijn.

CONCLUSIES EN EEN BLIK OP TOEKOMST

In dit proefschrift wordt op vele, uiteenlopende manieren getracht biomarkers te ontwikkelen welke mogelijk in de toekomst in een klinische setting op eenvoudige wijze toegepast kunnen worden ten einde de hoogrisico stadium II darmkankerpatiënten te identificeren. In alle studies werd een strategie gevolgd waarbij deze biomarkers werden opgespoord door te kijken naar de biologie van tumorgroei en -ontwikkeling en enkele van de belangrijkste processen zoals apoptose, proliferatie en interactie met het immuunsysteem te bestuderen. Deze studies hebben geleid tot een biochemische test waarin zowel apoptose als proliferatie gelijktijdig kunnen worden geanalyseerd, maar ook tot de ontwikkeling van tumor-immuunreactieve patronen met prognostische implicaties. De kennis verkregen in deze studies zal ons in de toekomst tevens helpen met de verdere ontwikkeling van de toepassing van therapeutische tumorvaccinatiestrategieën.

Alhoewel de resultaten veelbelovend zijn, zijn de door ons ontwikkelde biomarkerprofielen nog niet geschikt voor klinische toepassing. In hoofdstuk 2 wordt een vijf-stappenplan beschreven, ontwikkeld door Pepe *et al.*, dat kan worden gebruikt als leidraad voor de klinische introductie van een dergelijk biomarkerprofiel (39). Op dit moment bevinden wij ons wat betreft onze biomarkerprofielen pas in stappen 1 en 2 van dit plan, de zogenaamde pre-klinische 'exploratory' fase en de klinische toetsing en validatiefase. Er zijn verschillende problemen die nog aangepakt dienen te worden voor daadwerkelijk klinische introductie. De rol van tumormicrosatellietstatus bijvoorbeeld. Zoals al beschreven is duidelijk dat deze van invloed is

op prognose. Ook onze biomarkers lieten een evidente relatie zien met de tumor-microsatellietstatus, maar hoe deze interactie precies plaatsvindt blijft onduidelijk. Voor eventuele klinische introductie dient deze interactie te worden verhelderd. Er wordt gesuggereerd dat het mogelijk niet de microsatellietstatus op zich zelf is die de prognostische relatie beïnvloedt, maar de locatie van de tumor en de daarmee samenhangende embryologische oorsprong van het weefsel waaruit de tumor ontstaat, dan wel mate van mutaties die samenhangen met de microsatellietstatus (22, 23, 40, 41).

De resultaten van onze studies laten in ieder geval zien dat voor de ontwikkeling van prognostische biomarkers uitermate belangrijk is de biologische context van tumorgroei en -ontwikkeling als uitgangspunt te nemen. We hebben laten zien dat dit een gecompliceerd, uit meerdere stappen bestaand en op meerdere niveaus gereguleerd proces is. Door biomarkers te analyseren representatief voor een aantal van deze stappen zoals tumorcelapoptose, -proliferatie en interactie met het immuunsysteem binnen de tumoromgeving, kunnen tumorfenotypes worden ontwikkeld. Deze fenotypes zijn derhalve, meer dan de veelvuldig bestudeerde single markers, representatief voor de mate van tumoragressiviteit en daarom beter geschikt voor introductie als klinisch prognostische biomarkers. Op basis van dit proefschrift kan dan ook met vertrouwen worden gesteld dat de ontwikkeling van op biomarkers gebaseerde tumorfenotypen van grote klinische waarde is. Op basis van dergelijke fenotypen zal in de toekomst zeer accuraat het klinische gedrag van colontumoren kunnen worden voorspeld op basis waarvan uitspraken kunnen worden gedaan over de individuele prognose van patiënten. Derhalve zijn dergelijke fenotypen van grote klinische waarde en zullen in de toekomst veel bijdragen aan het bepalen van een optimale behandelstrategie, wat met name van groot belang is voor stadium II colonkankerpatiënten.

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Polycomb proteins EZH2, BMI1 and SUZ12 and histone modification H3K27me3 predict clinical outcome in colorectal cancer. A. Benard, I. Goossens-Beumer, A. van Hoesel, H. Horati, H. Putter, E. Zeestraten, C. van de Velde, P. Kuppen. Submitted for publication

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Acknowledgements

To start off I would like to state that I'm not a molecular biologist or a scientist for that matter, I am a clinician in training of becoming a surgeon and hopefully a scientist. The studies, which resulted in this thesis, could therefore not have been made possible without the help of many others, and most of all, very wise people. I would like to thank all of you who have been of such great help in this process. My supervisors for their patience, wisdom and guidance and my colleagues at the laboratory both in Leiden as well as in the JWCI for their inexhaustible support. Furthermore, everyone from the Surgical and Clinical Oncology Department and the Department of Pathology of the LUMC in Leiden and our colleagues at the Rechts an der Isar Klinikum and the TU in Munich who contributed one way or another to the completion of this thesis, thank you all very much.

Throughout this whole process I was lucky to receive the support of many people from both inside of the hospital (at the LUMC in Leiden, the GHZ in Gouda, at the Erasmus MC in Rotterdam and at the IJsselland Ziekenhuis in Capelle a/d IJssel) as well as outside the hospital (Rapenburg 120, Jaar 2001, my year club and many other friends) that enabled me to actually finish what I started. Thank you all very much.

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Finally, these acknowledgements would not be complete without a special thank you to Lot Ultee, Emmeline van Heukelem and Agnès Zeestraten. Whether it was as my best friend, my paranymp or my sister, any way you supported me throughout this process is much appreciated.

Dear mom and dad, I am so very grateful to you. Thank you for a great childhood, all of the opportunities you provided me with and your never ending support, love and understanding. Dear Godard, I am so happy I met you, thank you for support in something that was so important to me these past three years.

Curriculum vitae

Eliane Cornelia Maria Zeestraten was born on May 17th 1983 in Heerlen, the Netherlands. After graduating cum laude from the Gymnasium of the Bernardinus College in Heerlen in 2001, she started studying medicine that same year at the University of Leiden, the Netherlands. During her medical training she performed two research projects. The first one entitled; 'Pediatric outcome in Rhesus hemolytic disease treated with and without intrauterine transfusion' at the department of Neonatology in the LUMC in Leiden under the supervision of Dr. I. de Boer and Prof. Dr. F.J. Walther. The second one; 'Clinical impact of HLA class I expression in rectal cancer', at the department of Surgery in the LUMC under the supervision of Dr. P.J.K. Kuppen and Prof. Dr. C.J.H van de Velde. After graduating in 2007 she commenced with her medical rotations, obtaining her medical degree on September 27th 2009. Because of her great interest in academic research as well as the surgical profession, she choose to work on a PhD project at the Surgical Oncology research group of the department of Surgery in the LUMC. This PhD project that resulted in the current thesis was performed under supervision of Prof. Dr. C.J.H van de Velde and the co-supervisors Dr. P.J.K Kuppen and Dr. G.J. Liefers. During this four year period she spend 9 months at the Department of Molecular Oncology of the John Wayne Cancer Institute at Saint John's Health Center in Santa Monica, California, USA under the supervision of Dr. D.S.B. Hoon, performing additional studies on the clinical relevance of methylation profiles of apoptotic genes in rectal cancer. The results will be published in Apoptosis in the near future.

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After the four years she spent working on her PhD project she began her clinical training as a surgical resident (not in training) at the Groene Hart Hospital in Gouda, the Netherlands (head: Dr. R.F. Schmitz). In July 2012 she started her general surgical training at the Erasmus MC in Rotterdam, the Netherlands. (supervisor; Dr. B.P.L Wijnhoven) Currently, she is working as a surgical resident in training at the IJsselland hospital in Capelle a/d IJssel, the Netherlands. (supervisor; Dr. I. Dawson)

