Molecular prognostic and predictive markers of therapy response in sporadic colon cancer

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Caminante, son tus huellas
el camino y nada más;
caminante, no hay camino,
se hace camino al andar.
Al andar se hace camino
y al volver la vista atrás
se ve la senda que nunca
se ha de volver a pisar.

Antonio Machado

Wandelaar, jouw voetstappen
zijn de weg en niets anders;
Wandelaar, er is geen weg,
wandelend wordt de weg gemaakt.
Als je loopt maak je de weg
en als je naar achter kijkt, zie je de weg
die je nooit meer zal bewandelen.
(vrije vertaling)

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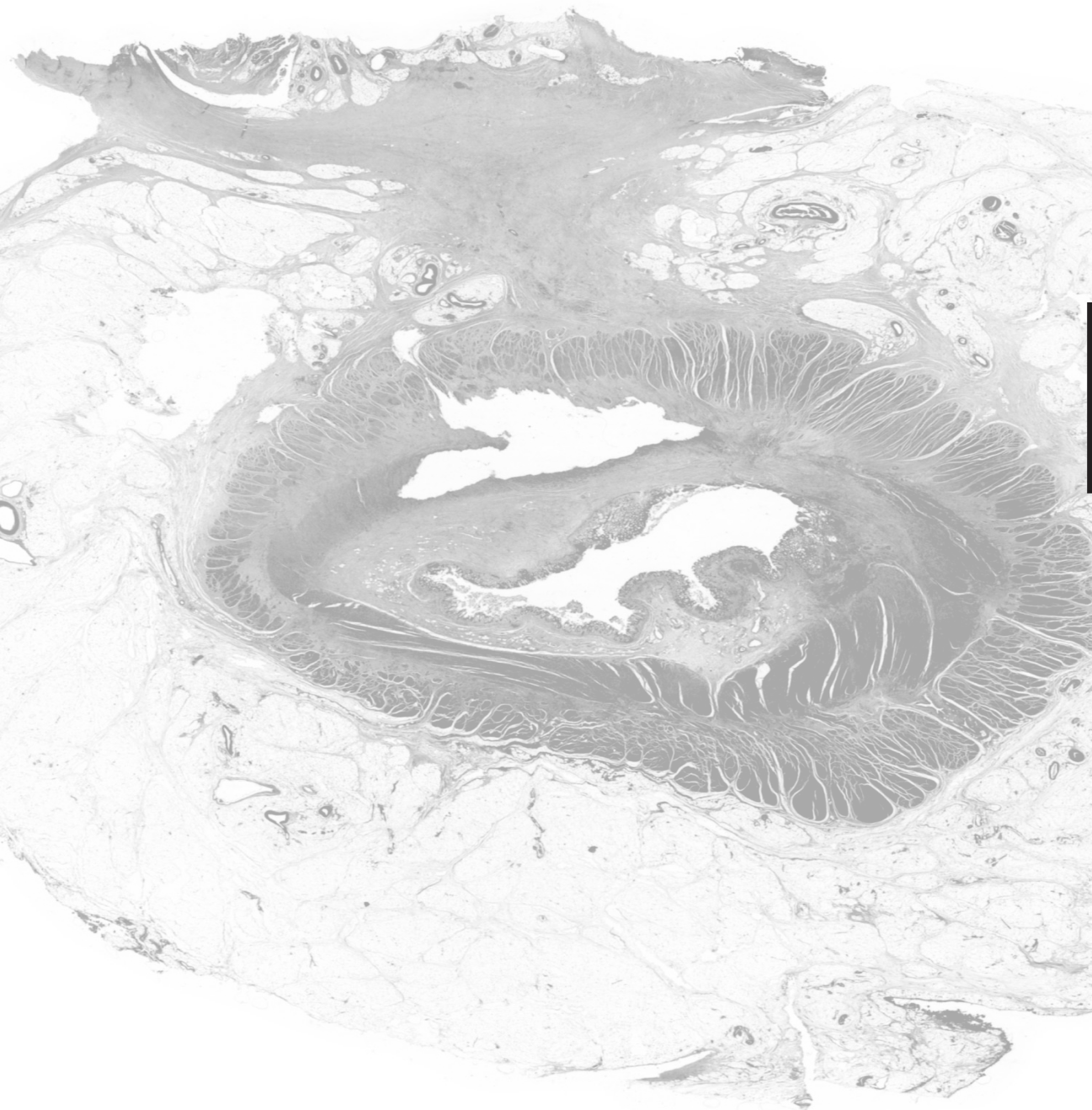
Outline of this thesis

The main goal of this thesis was to search for molecular prognostic and predictive markers of response to therapy in stage II and III sporadic colon carcinoma.

This thesis has two main parts: One corresponding to the search for predictive markers of response to therapy in stage III disease. The second part focuses on identifying prognostic markers in stage II and III sporadic colon cancer to distinguish different subgroups of patients needing different therapies.

In **chapter one** the epidemiology, pathophysiology and classification of colon cancer are shortly presented. In **chapter two**, the value of two different polymorphisms in the thymidylate synthase gene as predictive markers of response to 5-FU in stage III sporadic colon cancer patients is studied. **Chapters 3** and **4** deal with the value as predictive markers of SNPs in genes coding for enzymes involved in the metabolism of 5-FU and oxaliplatin and DNA damage repair in stage III colon carcinoma patients. In **chapters 5** and **6** the role of mutations in genes involved in known signalling pathways as prognostic markers is described. In **chapter 7** the "allelic state" of the *TP53* tumor suppressor gene in colon cancer and its role in disease prognosis are discussed. **Chapter 8** focuses on genomic aberrations linked to the *BRAF* V600E mutation. **Chapter 9** gives an overview of the technical issues of *KRAS* mutation detection assays before implementation in daily diagnostic practice. Finally, concluding remarks and future perspectives are presented in **Chapter 10**.



1

General introduction

1. Epidemiology of sporadic colon cancer

Colorectal cancer is one of the most frequent malignancies in the Western world. In the Netherlands the incidence of colorectal cancer reaches 10 000 new cases per year with a mortality of 3000 to 4000 patients every year[1,2]. Exclusion of rectal tumors leaves an incidence of 7000-8000 new colon cancer cases each year. Worldwide, approximately 1,2 million people developed colorectal cancer in 2008 and the disease related mortality was about 36%[3,4]. As more patients survive longer, the prevalence of colon cancer is increasing.

The disease affects slightly more men than women and sporadic colon cancer is considered to be a disease of the elderly with a median age at diagnosis of 70 years[1]. Several environmental and life style factors are suspected to increase colon cancer risk such as lack of physical activity, the consumption of red meat, cigarettes and alcohol. Other factors like intake of vegetables and fruit, a fibre rich diet or aspirin intake are considered possible protective factors for colorectal cancer[5,6].

Colon cancer can be subdivided in hereditary or sporadic depending on the presence or absence of familial genetic predisposition for the development of this type of cancer. Around 10-30% of the diagnosed colorectal cancers are considered to be hereditary, including cases of Familial Adenomatous Polyposis (FAP), Lynch syndrome previously known as HNPCC (Hereditary Non Polyposis Colorectal Cancer), *MUTYH* Associated Polyposis (MAP) and others[7]. The majority of the colon cancer cases are considered to be sporadic and form the focus of this thesis.

2. Colonic carcinogenesis

Colon adenocarcinoma emerges from normal colonic epithelium as a result of a sequence of genetic mutations and genomic alterations that lead to uncontrolled cell division and tumor formation. Such a sequence of events was first postulated by Vogelstein in the so-called Vogelgram, in which genetic alterations were schematically placed in the different morphologically recognisable phases of tumorigenesis. Grossly, there are two recognisable forms of sporadic colonic genetic instability; chromosomal instability (CIN) and the serrated form characterized by microsatellite instability (MIN)[3,8,9]. CIN and MIN were defined based on the insights from studies on FAP and Lynch syndrome respectively.

a. Chromosomal instability

The CIN pathway characterizes the majority of colon cancer tumors, around 80% of sporadic colon tumors develop through this pathway. The earliest identifiable lesion is the so called aberrant crypt focus (ACF)[10,11]. Certain mutations are already found in ACF like mutations in the *KRAS* and *APC* genes. Eventually, the dysplastic crypts will evolve into an adenomatous polyp[10]. Adenomatous polyps are benign but they can degenerate into malignant lesions. Although, polyps are frequently found in the large bowel of healthy individuals older than 50 years, only a relatively small fraction of polyps evolve into a malignant adenocarcinoma. Adenocarcinomas invade beyond the muscularis

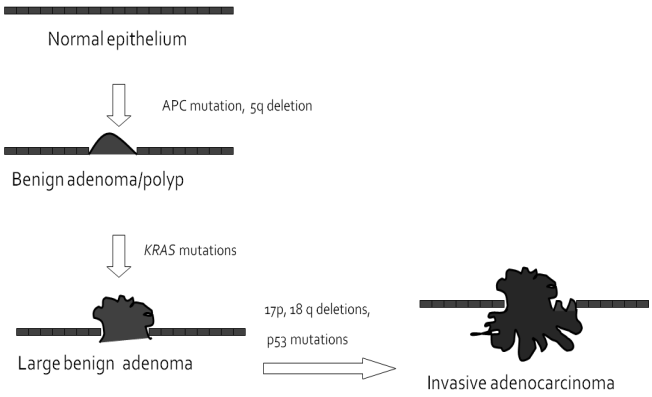


Figure 1: Schematic representation of the Vogelstein model of colonic carcinogenesis[15].

mucosae and can spread to regional lymph nodes and systemically. The transition from normal epithelium to benign adenoma and finally to malignant carcinoma is a relatively slow process that, in case of sporadic cancer can take several years. In the case of FAP, patients already develop thousands of adenomatous polyps by late adolescence. These FAP patients carry a germ line mutation in the *APC* gene; according to Knudson's hypothesis, in FAP only a second hit is needed to lose APC function[12]. During malignant transformation, the cells will get a growth advantage and start to divide uncontrollably through the sequential acquisition of several mutations in pivotal signal transduction pathways (*KRAS, TP53).* Genomic aberrations such as 17p and 18q deletions lead to genetic instability as shown in figure 1[13 14]. This model proposed by Vogelstein is still a valid model of colorectal carcinogenesis although several adaptations have been envisaged[15,16].

CIN tumors are characterized by numerical and structural chromosomal aberrations. CIN is probably caused by alterations in a myriad of systems like mitotic spindle checkpoints, centrosome regulation systems, DNA damage checkpoint genes, cell cycle regulators, telomeres and telomerases[11,17]. The majority of CIN tumors are aneuploid with highly aberrant DNA indexes in contrast to those tumors that are near diploid or pseudodiploid. The latter however, do show as well structural chromosomal aberrations although not numerical[18]. The prognostic value of ploidy in clinical practice has been a matter of discussion. However, recently it was established that DNA ploidy and CIN are prognostic markers[19-21]. Frequently, CIN is accompanied by mutations in known tumor suppressor genes like *TP53 (40-50%), SMAD4* (10-20%) and oncogenes such as *KRAS (30-50%) or PIK3CA (~20%)* [17].

b. Microsatellite Instability/ Serrated lesions

The identification of the Lynch syndrome evidenced that a different form of tumorigenesis could lead to colon cancer. The Lynch syndrome is the most common form of hereditary colon cancer. Patients with this syndrome have a very high risk of colon cancer and an increased risk of developing other tumors like endometrial or ovarian cancer. The adenoma carcinoma sequence differs at the genetic and histopathological level; Lynch syndrome tumors are driven through germ line mutations in care taker genes in contrast to the gatekeeper function that tumor suppressor genes such as *APC* hold[22]. In Lynch syndrome, germline mutation and secondary inactivation of *hMLH1, hMSH2, hMSH6* and *hPMS2* lead to loss of mismatch repair (MMR) and to

the incapacity of repairing specific DNA damage caused by the slippage of the DNA Taq polymerase. As a result, especially repetitive sequences, the so called microsatellites, become shorter or longer in tumor cells as compared to normal cells. Generally, these microsatellites are located outside coding regions, however, mistakes in microsatellites present in gene coding regions can be affected as well leading to the inactivation of certain genes like Tumor Growth Factor β receptor2 (*TGFβR2*) and Insulin growth factor like 2 receptor (*IGF2R*)[11]. A Lynch syndrome lesion has its sporadic counterpart in tumors with microsatellite instability, the so-called MSI-high or MSI-H tumors, mostly without gross chromosomal instability. MSI is seen in 15 to 20% of sporadic colon cancer cases and it is also caused by the inactivation of the MMR system. The latter occurs through hypermethylation of the promoter sequence of the *hMLH1* gene and not through mutation[23,24]. Phenotypically and clinically, MSI-H tumors are frequently right-sided tumors, poorly differentiated, with mucinous histology, with extensive intraepithelial lymphocytic infiltration and in general with a better outcome than other types of tumors[25,26]. The precursor lesion in this sequence to sporadic MSI-H tumor is the so called, sessile serrated polyp. An early mutation typical of this pathway is the *BRAF* V600E mutation which is subsequently followed by hypermethylation of the promoter region of the *hMLH1* gene accompanied with MIN and resistance to apoptosis[27,28]. Furthermore, the MSI-H tumors show extensive methylation of other genes like *HPP1, Era, MyoD1, RUNX3, CDKN2A* and the Methylated in tumor (MINT) sequences[29] annotated as the CpG Island Methylator Phenotype (CIMP). In order to study CIMP tumors in a standardized manner, an internationally well defined panel of markers is needed; however, the best gene panel to classify this subtype of tumor is still a matter of discussion[30-34].

3. Signal transduction pathways in colon cancer pathogenesis

Many cellular signaling pathways become deregulated in tumors through mutational activation or inactivation of the genes/proteins implicated in such pathways. Signaling pathways are complicated networks of proteins with much interaction as shown in figure 2. Certain pathways are preferentially disrupted in colon cancer, making the proteins involved, drugable targets for new therapies.

a. Wnt/β-catenin signaling pathway

The Wnt signaling pathway plays an essential role in the development and maintenance of intestinal epithelium. Deregulation of this pathway is observed in many cancer types and particularly in colon cancer. Briefly summarized, the pathway acts as follows; upon Wnt activation, β-catenin translocates to the nucleus where it acts as a transcription factor for several target genes like *c-myc* and *cyclin D1*. If Wnt is not activated, β-catenin is targeted for degradation via a complex formed among others by Adenomatous Polyposis Coli (APC) and Glycogen Synthase Kinase 3β (GSK3β). The *APC* gene is frequently mutated in colorectal cancers. Mutations give rise to a truncated protein leading to a decreased degradation of β-catenin, its accumulation in the nucleus and the constitutive activation of Wnt target genes stimulating cell division and proliferation[35,36].

b. EGFR/KRAS/BRAF/MAPK pathway

The Epidermal Growth Factor receptor (EGFR) signaling pathway is essential for epithelial cell growth. EGFR is a tyrosine kinase that signals downstream via KRAS and BRAF to the MAP kinases finally to the nucleus where it stimulates cell division and proliferation[37]. EGFR can also signal through the Phosphatidyl Inositol 3 kinases (PI3K) pathway with the AKT kinase and finally mTOR as downstream targets.

The whole pathway is altered in more than 50% of all colon cancer cases[38]. Moreover, it is an important target for cancer therapy; monoclonal antibodies blocking EGFR activity currently form part of the targeted therapy in metastatic colon cancer. However, patients, with mutations of downstream effector molecules do not respond to this therapy[39-45].

c. p53 cell cycle checkpoint pathway and apoptosis pathway

Although, p53 is not involved in a signal transduction pathway, it plays an important role in colon carcinogenesis as over 50% of colon tumors inactivate p53. This inactivation is considered to be a late event in the adenoma carcinoma sequence and correlates with chromosomal instability.

p53 is a transcription factor with key roles in essential pathways for normal cellular physiology. It is implicated in DNA damage repair, apoptosis, senescence, cell cycle checkpoints, cell proliferation and cytoskeletal characteristics[46].

Of importance for colon carcinogenesis is p53 function of sensing DNA damage and causing cell cycle arrest at G2 phase. When p53 is activated it will transcribe many downstream targets like *CDKN1A* and *GADD45* which inhibit cyclin dependent kinases causing subsequently cell cycle arrest. Furthermore, when DNA damage is not repairable, p53 will direct the cell to apoptosis by activating *BAX*. *TP53* is located on chr17p and is one of the genes very frequently inactivated in human cancers leading to resistance to apoptosis and accumulation of DNA and genomic aberrations[47,48].

d. TGFβ/ BMP pathway

The Transforming Growth Factor β (TGFβ) superfamily consists of the TGFβ and Bone Morphogenetic Protein (BMP) subfamilies. TGFβ is involved is several cellular processes like proliferation, differentiation, migration and apoptosis. It seems that TGFβ has a dual role stimulating both cell growth and growth arrest depending on the targets it activates. Its role in carcinogenesis is therefore complex acting as both tumor suppressor gene and oncogene[49]. The tumor suppressor activity is driven through Smad signaling. Upon ligand binding to the TGFβ receptor, intermediate factors like Smad2 and Smad3 will become phosphorylated and will form a complex with Smad4 which will in turn translocate to the nucleus and inhibit *c-myc* transcription and activate cyclin associated proteins like cyclin D1 and p21. Other members of the Smad family like Smad6 and Smad7 act as “inhibitors” of the TGFβ signaling by interfering with the activation of the effector Smads. Smad7 is activated by TGFβ itself representing a negative feedback loop for the pathway regulation. Contrasting with this growth suppressive function, TGFβ can enhance invasion capacity of tumor cells and facilitate metastasis, considered to be oncogenic events. The switch between tumor suppression effects and tumor progression effects is quite complex and partly due to the decreased signaling through *TGFβR2* and Smad molecules also favoring MAPK signaling[49]. In colon cancer, *TGFβR2* is

found mutated in up to 80% of MSI-H tumors and 15% of MSS tumors[35 50].

As TGFβ, BMPs also signal through Smad proteins and act as a tumor suppressor gene in colon carcinogenesis. Once a BMP ligand is bound to the BMP receptors, these will become phosporylated and in turn will phosphorylate Smad1, Smad5 and Smad8 which will associate with Smad4 and enter the nucleus where they regulate gene transcription[51]. BMP2 seems to act in colonic epithelium as a tumor suppressor promoting apoptosis of epithelial cells[52]. BMPs are involved in colon carcinogenesis as suggested by the mutations in BMP receptor type Ia (*BMPR1A*) in the pathogenesis of juvenile polyposis[53]. Moreover, in sporadic colon cancer, the BMP pathway is inactivated in 70% of the cases through loss of Smad 4 or loss of BMPR2 expression. In sporadic colon cancer, the BMP signaling seems to have a role in tumor progression rather than tumor initiation[51].

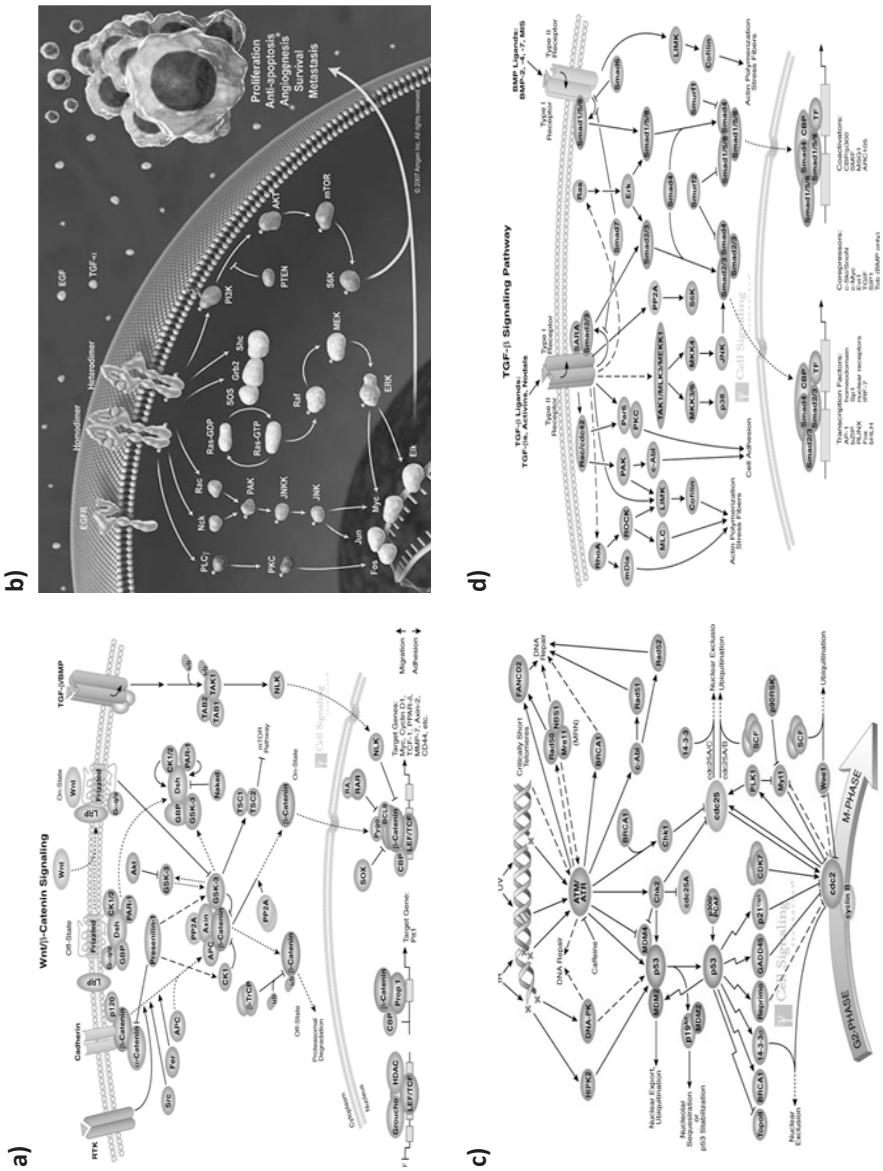


Figure 2: Signaling pathways in colon cancer pictures from cell signaling technology (www.cellsignal.com viewed Feb 14, 2011) a) Wnt/β-catenin pathway b) EGFR/KRAS/ BRAF/MAPK and PI3K pathways (adapted from Allison[54]) c)p53 cell cycle checkpoint pathway (www.cellsignal.com viewed Feb 14 2011) d) TGFβ/BMP pathway (www.cellsignal.com viewed Feb 14, 2011).

4. Current classification and therapy of sporadic colon cancer

Clinicopathologically, colon cancer is classified in different stages according to a stepwise analysis of items such as the extent of colonic wall infiltration, the absence or presence of lymph node metastasis and the existence of distant metastasis. Nowadays, other factors are also being taken into account like venous, lymphatic or perineural infiltration, tumor budding, proportion of stroma and tumor grading, as these parameters have shown to influence prognosis as well [6,55-57]. In daily clinical practice, the TNM classification of the American Joint Committee on Cancer (AJCC) and the "Union Internationale Contre le Cancer" (UICC) is used (Table 1).

At diagnosis 14% of the patients have stage I disease, 28% stage II, 37% stage III and 21% stage IV. Prognosis is frequently measured as five-year survival. Five-year survival is stage dependent and varies from over 90% in stage I to less than 5% in stage IV disease [3,58].

The treatment of colon cancer depends mainly on disease stage at diagnosis. Patients with stage I and II have localized disease and are therefore considered cured after surgery whereas patients with stage III disease will receive adjuvant chemotherapy as the disease has spread outside the bowel into the lymphatic system. In general, stage IV patients are considered not curable because of the spread of the disease to different organs and tissues. These patients will therefore receive palliative treatment.

Stages II and III form the focus of this thesis as the disease at these stages is potentially curable. Much benefit can be obtained from a molecular subclassification leading to a more patient tailored therapy.

In Europe, adjuvant chemotherapy for stage III consists on 5-fluorouracil (5-FU) or its derivate capecitabine in combination with oxaliplatin during six months; the so called FOLFOX (5-FU and oxaliplatin) or CAPOX (XELOX) (capecitabine and oxaliplatin) regimes[6]. The use of adjuvant chemotherapy in stage III is nowadays widely accepted as it has been shown to reduce cancer related death in 29% as 5-FU monotherapy and even further as combination therapy with oxaliplatin [1,59-62].

The value of adjuvant chemotherapy in stage II remains however more controversial[58,63,64]. Although several international trials have failed to show any benefits of this treatment in stage II patients, the recurrence rate at this stage, over 15%, is relatively high for localized disease [58]. Therefore, a new subgroup of stage II patients at high risk of a relapse has been defined as stage II disease with either one of the

following characteristics; T4 tumors, poorly differentiation, less than 10 lymph nodes yield in the surgical resection specimen (in the Netherlands) or a clinical presentation with bowel obstruction or perforation. Patients classified as high risk stage II receive the same adjuvant chemotherapy scheme as stage III patients do.

Table 1: AJCC/UICC classification of colon carcinoma [3].

T primary tumor	**TNM**	**Stage**
T1 tumor invades submucosa		
T2 tumor invades muscularis propria	T1-T2 N0	Stage I
T3 tumor growths through muscularis propria into subserosa	T3 N0	Stage II A
T4a tumor penetrates visceral peritoneum	T4a N0	Stage II B
T4b tumor invades other adjacent tissues or organs	T4b N0	Stage II C
N regional lymph nodes		
N0 no regional lymph nodes affected	T1-2 N1	Stage III A
N1 one to three regional lymph nodes affected	T1-2 N2	Stage III B
N2 more than three lymph nodes affected	T3 N1	Stage III B
	T4 N1	Stage III C
	T3-4 N2	Stage III C
M distant metastasis		
M0 no distant metastasis present	Any T any N M1	Stage IV
M1 distant metastasis present		

Targeted therapies have made their entrance in colon cancer treatment but their use remains limited to metastatic colon cancer stages. Compounds like bevacizumab targeting Vascular Endothelial Growth Factor (VEGF), the mouse anti human monoclonal antibody cetuximab or the humanized antibody panitumumab both targeting EGFR have shown survival benefit in stage IV patients with no mutations in downstream effector molecules [40,65,66]. The benefit of these therapies in earlier stages of the disease is currently being studied. The preliminary results of the NSABP-Protocol 08 clinical trial comparing FOLFOX alone or in combination with bevacizumab in the adjuvant setting show, however, no survival improvement in stage II and III colon cancer patients and therefore the administration of bevacizumab as adjuvant therapy is not advised at this point [67-69].

In conclusion, there is need for a more accurate classification of patients who are likely to benefit from adjuvant chemotherapy and patients who are not. This classification could be based not only on clinicopathological features but also on molecular profiles

of the tumors. These molecular markers, responsible for different phenotypes and clinical behaviors, could be used in the future as determinants of outcome or markers of response leading to personalized therapy and management of the disease[3,9,26,58,64,70,73]. The main goal of this thesis is to find these molecular markers of prognosis or of response to therapy in stage II and III disease. The following paragraphs describe the strategy that has been followed to this purpose.

5. Pharmacogenomics and predictive markers of therapy response

As the human genome has been completely sequenced, it has become clear that DNA variability is even larger than originally thought. Single nucleotide polymorphisms (SNPs) or, in other words variation in one base pair, constitute the most frequent variation in the DNA sequence with an estimated frequency of one polymorphism in hundred nucleotides. Other variation types have been described as well, such as short tandem repeats (STRs) and copy number variations (CNVs). However, the exact consequences of this kind of variation in gene expression and protein function are less understood.

SNPs can reside in coding as well as in non coding regions, besides, SNPs can be non synonymous and synonymous depending on whether they cause an aminoacid substitution or not, respectively. The latter can cause however, discrete alterations in protein function like slightly different protein folding or altered expression through the use of a less effective codon [74].

SNPs are present throughout the whole genome influencing the expression of several proteins. Enzymes involved in drug metabolism are no exception to this genetic variation. Pharmacogenomics is the discipline that studies the effect of genetic polymorphisms in the effectiveness of certain drugs. It can be hypothesized that variation in genes coding for proteins involved in the metabolism of chemotherapeutic agents as well as in DNA repair, or genes coding for target proteins of chemotherapeutic drugs are potentially good candidates for predicting response of a patient to a certain chemotherapeutic drug, becoming a predictive marker or marker of response[75-78]. In other words a predictive marker is a patient's pheno and genotype determining the patient' s response to a certain drug.

In colon cancer, several molecules involved in the metabolism of 5-FU and oxaliplatin as well as the target protein of 5-FU and DNA damage repair proteins are subjects of pharmacogenomic investigation. For new targeted therapies, like EGFR blocking agents, mutations in downstream effector molecules like *KRAS* and *BRAF* are predictive markers of response to EGFR blockers [40,79].

6. Prognostic markers

Prognostic markers are tumor related or patient related characteristics that identify the tumor as aggressive or less aggressive.
There are several possible approaches to identify new prognostic markers. One is to study the prognostic value of mutations in known genes involved in e.g. signal transduction pathways, apoptosis, cell cycle or DNA repair. Other strategies search the whole genome of the tumor or its expression signature to identify profiles that are associated with a good or poor prognosis.

a. Genetic mutations

Certain mutations are typically found in specific types of cancer[48]. In the case of colon cancer, mutations in *APC and KRAS* have been extensively found [80]. As previously mentioned, with the introduction of targeted therapies, mutations in genes such as *KRAS* and probably *BRAF* have become very important as predictive markers of response in stage IV colon carcinoma [40,43,79]. However, their prognostic value in earlier disease stages is not clear yet[80,81] and it is a subject of ongoing research. Nevertheless, there is some evidence towards a prognostic role in colorectal cancer for mutations in *BRAF* and *PIK3CA* as they have been associated with a poor prognosis in MSS colon tumors and in rectal cancer respectively[82,83]
Gene mutations might be used to classify tumors more accurately according to their molecular signature instead of their histopathological phenotype. Tumor heterogeneity can however pose a serious problem to this aim. Intratumor heterogeneity has been recognized previously; however, the biological and clinical implications of this heterogeneity are still largely unknown. However, tumor initiation and progression is seen, quite simply, as a linear succession of acquisition of mutations and other genetic hits leading to clonal expansion. Tumor cells are constantly changing and adapting to their microenvironment and not all tumor cells are exposed to exactly the same microenvironment as they receive different external signals (growth factors, oxygen, blood supply, inflammatory cells). Tumors therefore, are evolving in different directions giving rise to different clones within a single tumor with potentially different behaviours. Clinical cancer research is limited by the fact that patient material represents the tumor status at a given time, namely time of diagnosis and surgery. Therefore tumor plasticity is not a very well studied subject[84,85]. Nevertheless, it has been shown that

tumor cell populations are not always monoclonal [86] and that several cell lines with different genetic abnormalities can co-exist in the same tumor [87].

Tumor heterogeneity also constitutes a technical challenge. Laser capture based microdissection and cell separation by flow cytometry or magnetic beads can be useful in obtaining homogeneous tumor cell populations. However, these are time consuming techniques not really feasible to study large cohorts of patients [13].

In the context of tumor heterogeneity another problem can be seen, the way to interpret results clinically from very sensitive analyses that are able to detect very small populations of tumor cells[88]. The significance of 1% mutated cells in a tumor for decision making in targeted therapy remains unknown. Moreover, discrepancies in mutation patterns between primary tumors and metastatic clones have also been described [38]. This issue can complicate the use of targeted therapies and the implementation of molecular marker testing for therapy decision making [89].

b. Whole genome analysis in sporadic colon cancer

Whole genome research has been widely applied in colon cancer research. Results have been obtained using different platforms like gene expression arrays, comparative genomic hybridization (CGH), array CGH and more recently, high density SNP arrays and next generation sequencing. Results from expression arrays are able to discriminate between different disease stages, mutational phenotypes, lymph node positivity and prediction of disease recurrence [90-95]. Recently, a prognostic signature for stage II and III colon cancer containing eighteen genes was published[96]. Clinical validation and regulators approval are difficult to obtain before these tests can be used in daily clinical practice.

Several genomic regions have been consistently identified to be altered in colon cancer such as losses of chromosomes 17p, 18, 4p, 8p and 14q and gains of 8q, 13q, 20, 7p, 17q, 1q, 11, 12p and 19 [13,97-104]. Moreover, these genomic alterations have been associated to colon cancer progression [13]. However, identifying the genes or regulating sequences implicated in these altered genomic regions has turned out to be more difficult than initially thought [105].

Despite all the effort, until date, only two molecular markers are accepted as prognostic markers in colon cancer, namely chr.18q loss and MIN [55,106]. The existence of a “genomic signature” responsible for a more aggressive phenotype is a subject of ongoing investigation.

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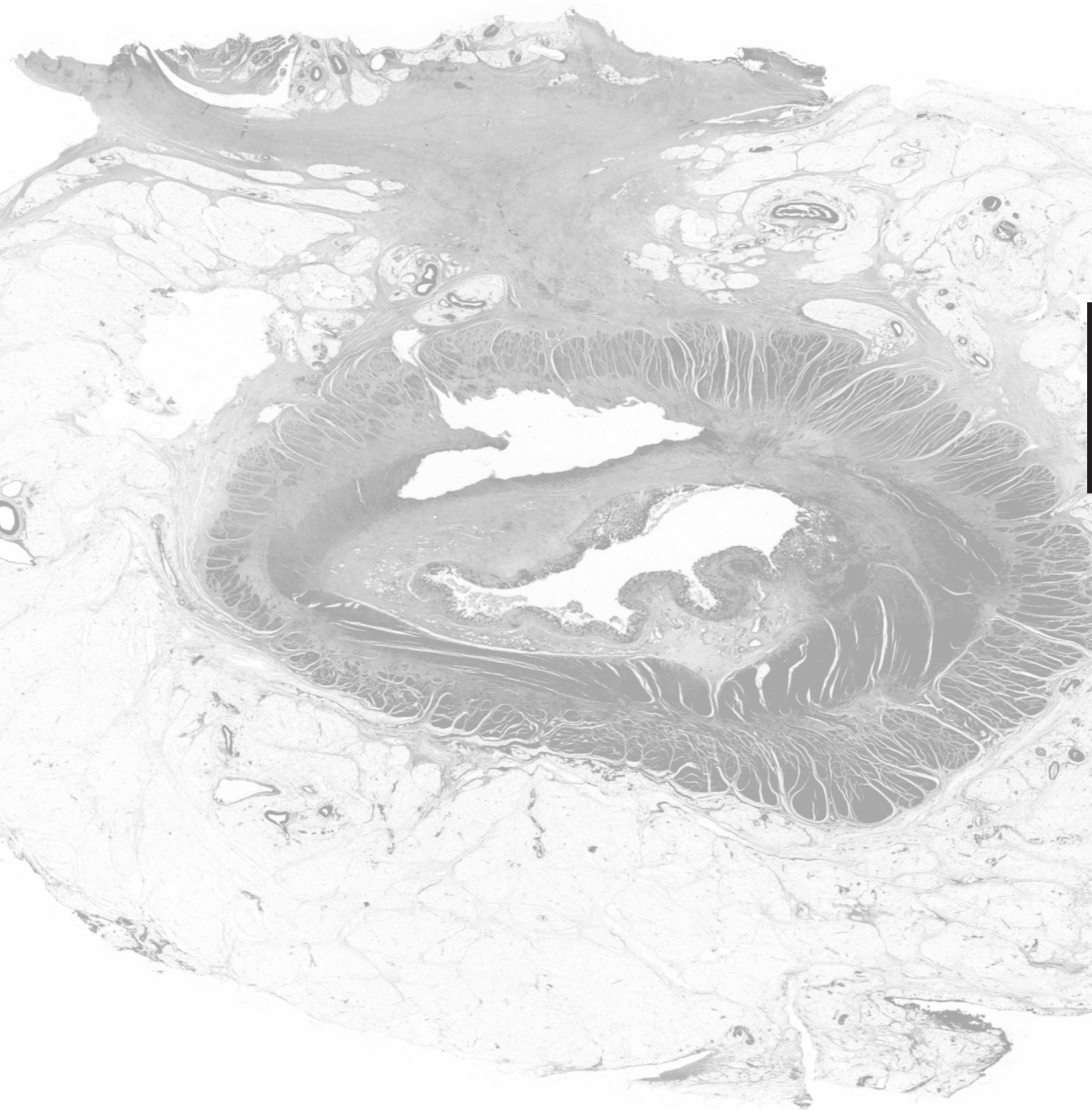

2

TYMS gene polymorphisms are not good markers of response to 5-FU therapy in stage III colon cancer patients

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Abstract

Although the predictive and prognostic value of thymidylate synthase (*TYMS*) expression and gene polymorphism in colon cancer has been widely studied, the results are inconclusive probably because of methodological differences. With this study, we aimed to elucidate the role of *TYMS* gene polymorphisms genotyping in therapy response in stage III colon carcinoma patients treated with 5-FU adjuvant chemotherapy.

Two hundred and fifty one patients diagnosed with stage III colon carcinoma treated with surgery followed by 5-FU based adjuvant therapy were selected. The variable number of tandem repeats (VNTR) and the single nucleotide polymorphism (SNP) in the 5'untranslated region of the *TYMS* gene were genotyped.

There was a positive association between tumor T stage and the VNTR genotypes ($p=0.05$). In both univariate and multivariate survival analysis no effects of the studied polymorphisms on survival were found. However, there was an association between both polymorphisms and age. Among patients younger than 60 years, the patients homozygous for 2R seemed to have a better overall survival, whereas among the patients older than 67 this longer survival was seen by the carriers of other genotypes.

We conclude that the *TYMS* VNTR and SNP do not predict response to 5-FU therapy in patients with stage III colon carcinoma. However, age appears to modify the effects of *TYMS* polymorphisms on survival.

Introduction

5-Fluorouracil (5-FU) is the chemotherapeutic drug of choice in the treatment of colon cancer. 5-FU causes cell death through two different mechanisms [1]. One mechanism is the incorporation of fluorouracil triphosphate (FUTP) into RNA causing disruption of normal RNA processes. The second mechanism of action consists on inhibition of thymidylate synthase (TS). TS provides the sole de novo source of thymidylate for DNA synthesis, thus TS inhibition causes depletion of nucleotides disrupting DNA synthesis and repair. Besides, it also causes DNA damage through misincorporation of deoxyuracil triphosphate (dUTP) into the DNA strand [1]. The fact that enhanced TS protein expression has been described as a mechanism of acquired 5-FU resistance [2] supports the thesis that TS inhibition is the main mechanism of action of 5-FU.

Because of its role as potential main target of 5-FU, TS has been widely studied as a molecular maker of therapy response in colorectal cancer, without conclusive results. Several studies have focussed on quantitation of TS protein by immunohistochemistry (IHC) [3-12] or mRNA expression [8,13-22] in tumors and metastasis whereas others have focussed on gene polymorphisms genotyping [6,11,23-39]. Besides technical differences, heterogeneity in patient selection also plays a role in the lack of consistency between results. Many studies for instance have included patients with rectal cancer [26,32,33,38], while these are treated differently than colon cancer. Furthermore some reports described heterogeneous cohorts of patients including all disease stages and patients who did not receive 5-FU based adjuvant therapy at all [24,26,32,37,38]. Results are therefore frequently contradictory [40].

We have recently reported the reliability of different methods for *TYMS* typing, like genotyping of three known gene polymorphisms (see figure 1), *TS* protein expression quantitation, *TYMS* gene amplification and loss of heterozygosity in predicting 5-FU therapy response [41]. From these results, it seemed that genotyping of the 5'untraslated region polymorphism of the *TYMS* gene was more reliable for predicting response to therapy than protein expression, as determined by IHC and than genotyping the rest of polymorphisms in the 3'UTR.

The aim of this study was to determine the value of the *TYMS* gene 5'UTR polymorphisms as a possible molecular marker for 5-FU response in a well defined, homogeneous population of stage III colon cancer patients who had been treated with 5-FU based adjuvant chemotherapy.

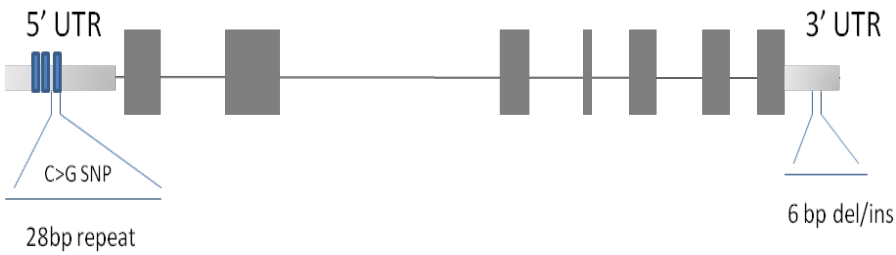


Figure 1: Schematic representation of the *TYMS* gene with known polymorphisms in 5' untranslated region (5'UTR) and 3' UTR. On the 5'UTR the 28 bp repeat with the SNP in the third repeat. Two or three repeats are the most frequent alleles in the Caucasian population. On the 3'UTR a 6bp long deletion/insertion.

Materials and methods

Patients

All patients (n=251) were stage III colon carcinoma patients treated with surgery followed by 5-FU based adjuvant chemotherapy between 1995 and 2004 in four different hospitals in the Eindhoven area in the south of the Netherlands.
Two hundred forty two patients (96.4%) received 5-FU in combination with leucovorin following the Mayo regime, 4 patients (1.6%) had 5-FU plus levamisole and finally 5 patients (2%) received capecitabine.
Routine histopathological diagnoses were performed in a central laboratory, the PAMM laboratory for Pathology in Eindhoven. Epidemiological data and tumor characteristics of all patients included were extracted from the Eindhoven Cancer Registry of the Comprehensive Cancer Centre South (IKZ, the Netherlands). Follow up information was obtained from the medical records of these patients. The research protocol was approved by the Scientific Committee of the Catharina Hospital Eindhoven.

Methods

VNTR typing
DNA was obtained after proteinase K digestion of 5 sections of 5 µm from formalin fixed paraffin embedded (FFPE) blocks with normal colonic tissue. Subsequently, the tissue digest was purified with HPPTP purification kit for genomic DNA (Roche diagnostics, Almere, the Netherlands). PCR for the VNTR was performed using the following primers: (forward) 5'gcg gaa ggg gtc ctg cca3' and (reverse) 5'tcc gag ccg gcc aca ggc at3'. The reaction was performed in 50µL final volume as described elsewhere[42]. PCR products were separated by electrophoresis on a 2% agarose gel. The expected product sizes were 107 bp for the 2R allele and 135 bp for the 3R allele.

SNP genotyping
Subsequently, the previously obtained PCR products were digested by HaeIII restriction enzyme during one hour at 37°C (New England Biolabs, Ipswich, United Kingdom). The G to C base change removes a HaeIII restriction site present at position 12 of the second 28 bp repeat of the 3R allele. PCR products of carriers of the G allele will be digested giving an additional shorter band of 66 bp after gel electrophoresis on a 3% agarose gel.

Statistical analysis

Statistical analyses were performed using SPSS software package for Windows (Chicago, Il., U.S.A.). Categorical data were analyzed by means of a chi-square or Fischer's exact test. To study the difference in median age between the different VNTR and SNP genotype groups, age was used as a continuous variable to perform a Kruskal-Wallis test. After this, age at diagnosis was categorized according to tertiles for further analyses.

To study the effects of the different polymorphisms on 5-FU response, survival analysis was used. The univariate survival analysis was performed using the Kaplan Meier test. Differences between survival curves were tested for significance by the Log-rank test. Overall survival (OS) was the time between surgery and death discriminating between death because of colon cancer or because of other reasons when this was specified in the medical records. Disease free survival (DFS) was the time between surgery and disease progression. Cancer specific survival (CSS) was defined as the time between surgery and death because of colon cancer. Cox proportional hazards regression analysis was used for multivariate survival analyses. All tests were two-tailed and $p<0.05$ was considered to be statistically significant.

Results

Clinicopathological characteristics

Patient and tumor histopathological characteristics are shown in table 1. All patients had positive lymph nodes and no recognizable distant metastasis at time of diagnosis. 10 patients (4 %) developed distant metastasis within the first four months following surgery.

Median follow-up was 47 months (range 2-133 months). 122 patients (49%) were still alive at the end of the follow up period, 30 patients (12%) were alive but had had disease progression, 80 (32%) died due to cancer related causes and 17 patients (7%) died due to non cancer related causes according to the medical records. Finally, medical records of two patients were incomplete and their follow-up status was unknown.

VNTR distribution

VNTR distribution and association with studied variables is shown in table 1. Distribution of the VNTR in the population studied followed Hardy Weinberg equilibrium. There was a significant association between tumor T stage and VNTR alleles. Patients homozygous for the 2R allele had significantly more frequently low T stages than did heterozygous and homozygous 3R (p=0.05).

There was, further, a significant association between age at diagnosis and the three genotypes. Median age in the group with the 3R/3R genotype was significantly lower than median age in the 2R/2R and in the 2R/3R group; 61 years vs. 64 and 65 respectively (H=14.633 p=0.001 99%CI 0.000-0.001). To further study the association between age at diagnosis and genotypes and their role in survival, we categorize age in three different groups according to tertiles. These tertile groups corresponded in our study population to the following age categories; younger than 60 years, between 60-67 years, and older than 67 years, respectively. There was a significant relationship between the three genotypes and the three age categories (p=0.02).

SNP distribution

Two hundred and thirteen out of 251 patients had enough PCR product available to study the G>C SNP present in the second repeat of the 3R allele.

Frequencies of the different SNP alleles in our patient population were in agreement with the in the literature published frequencies and are shown in table 2. There was

no significant association between the different SNP alleles and any of the categorical variables tested.

Age was tested as a continuous variable and there was a significant association with the SNP genotypes (H=15.135 p=0.01 99%CI 0.006-0.01). Median age in the 3G/3C group was 53,5 years, whereas all the other genotype groups had a median age greater than 60 years (figure 2). When age was categorized according to tertiles, a positive trend was seen towards an association between age tertiles and the SNP (p=0.06).

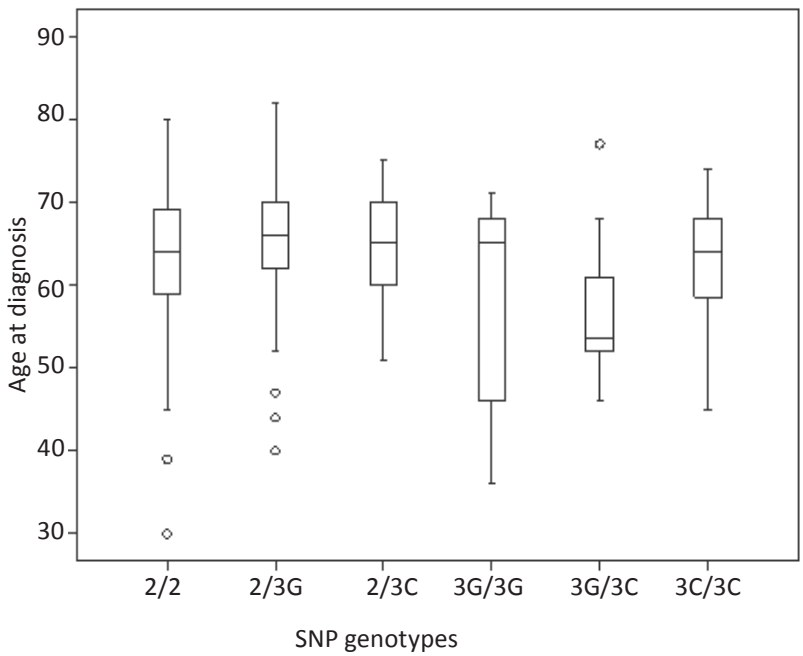


Figure 2: Age distribution according to SNP genotypes (Kruskal-Wallis H=15.135 p=0.01 99%CI 0.006-0.0.

Categorization into high and low TS expression

Based on the effects of the VNTR in TS protein expression as described in the literature, our patient population was divided in two putative categories low and high TS expression, according to the genotypes found: homozygous 2R and carriers of the 3R allele (3R/3R, 2R/3R), respectively [30,31,34,42,43].

When additionally the SNP genotypes were included, patients could be divided in the following groups: putative high TS expression as carriers of the G allele (3RG/3RG, 3RG/3RC, 2R/3RG) and putative low TS expression as carriers of the C allele plus the

Table 1: Patient's characteristics. Histopathological features of the tumors in relation to VNTR distribution.

Demographic & Histopathological characteristics	Total N (%)	2R/2R 59 (23)	2R/3R 128(51)	3R/3R 64 (26)	p-value
Gender					
Female	112 (45)	23 (40)	58 (45)	31 (48)	0.6
Male	139 (55)	35 (60)	70 (55)	33 (52)	
Age					
First tertile ≤59	78 (31)	17 (29)	31 (24)	30 (47)	0.02
Second tertile 60-67	88 (35)	21 (36)	46 (36)	21 (33)	
Third tertile >67	85 (34)	20 (35)	51 (40)	13 (20)	
Median age	64	64	65	61	
Tumor location					
Right	133 (54)	29 (50)	73 (57.5)	31(51)	0.5
Left	114 (46)	29 (50)	54 (42.5)	30 (49)	
T stage					
T1	1 (0.4)	1 (2)	0 (0)	0 (0)	0.05
T2	22 (8.6)	9 (15)	9 (7)	4 (6)	
T3	183 (73)	44 (76)	94 (73)	45 (70)	
T4	45 (18)	4 (7)	25 (20)	15 (24)	
Positive lymph nodes					
1-3 N1	135 (70)	32 (70)	67 (70.5)	36 (69)	0.9
≥ 4 N2	58 (30)	14 (30)	28 (29.5)	16 (31)	
Differentiation grade					
Well differentiated	28 (12)	6 (11)	16 (13)	6 (10)	0.6
Moderated	148 (61.6)	36 (65)	69 (58)	39 (64)	
Poor	62 (26)	12 (22)	34 (29)	16 (26)	
Undifferentiated	1 (0.4)	1 (2)	0 (0)	0 (0)	

Table 2: Distribution of SNP genotypes according to histopathological features.

N (%)	2R/2R 59 (28)	2R/3R		3R/3R			p-value
		2R/3RC 50 (23)	2R/3RG 53 (25)	3RC/3RG 14 (7)	3RC/3RC 20 (9)	3RG/3RG 17 (8)	
Gender							
Female	24 (41)	24 (48)	21 (40)	6 (43)	14 (70)	7 (41)	0.3
Male	35 (59)	26 (52)	32 (60)	8 (57)	6 (30)	10 (59)	
Age							
First tertile≤59	17 (29)	12 (24)	11 (21)	10 (72)	5 (25)	7 (42)	0.06
Second tertile 60-67	21 (36)	21 (42)	20 (38)	2 (14)	10 (50)	5 (29)	
Third tertile >67	21 (36)	17 (34)	22 (41)	2 (14)	5 (25)	5 (39)	
Median age	64	65	66	64	53.5	65	
Tumor Location							
Right	29 (49)	25 (50)	35 (67)	8 (57)	9 (47)	10 (62.5)	0.4
Left	30 (51)	25 (50)	17 (33)	6 (43)	10 (53)	6 (37.5)	
T stage							
T1	1 (2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.2
T2	9 (15)	3 (6)	4 (7.5)	0 (0)	1 (5)	3 (18)	
T3	45 (76)	35 (70)	40 (75.5)	11 (79)	15 (75)	8 (47)	
T4	4 (7)	12 (24)	9 (17)	3 (21)	4 (20)	6 (35)	
N stage							
N1	33 (70)	28 (72)	22 (61)	9 (75)	11 (79)	8 (50)	0.5
N2	14 (30)	11 (28)	14 (39)	3 (25)	3 (21)	8 (50)	
Differentiation grade							
Well differentiated	6 (11)	4 (9)	7 (14)	1 (8)	4 (20)	1 (6)	0.7
Moderated	37 (66)	27 (59)	31 (63)	10 (83)	12 (60)	9 (53)	
Poor	12 (21)	15 (33)	11 (23)	1 (8)	4 (20)	7 (41)	
Undifferentiated	1 (2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	

2R homozygous (2R/2R, 2R/3RC, 3RC/3RC). 60% of the patients were categorized as putative low expression vs. 40% putative high expression.

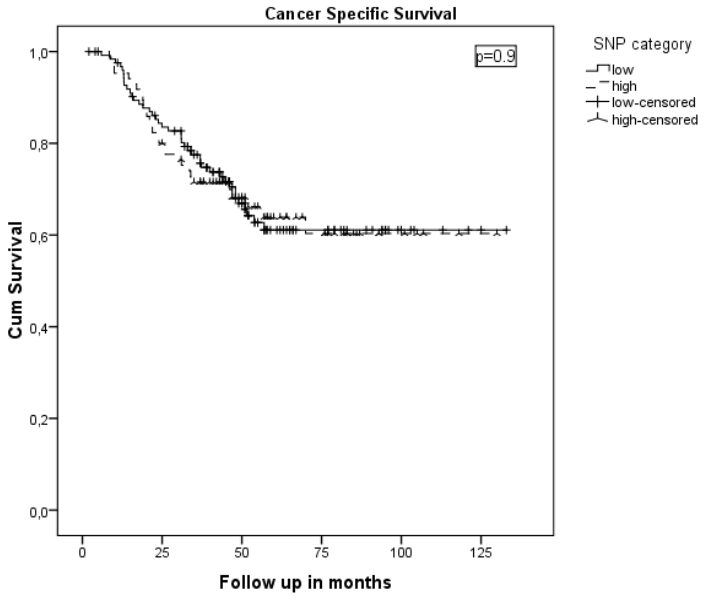
Survival analysis

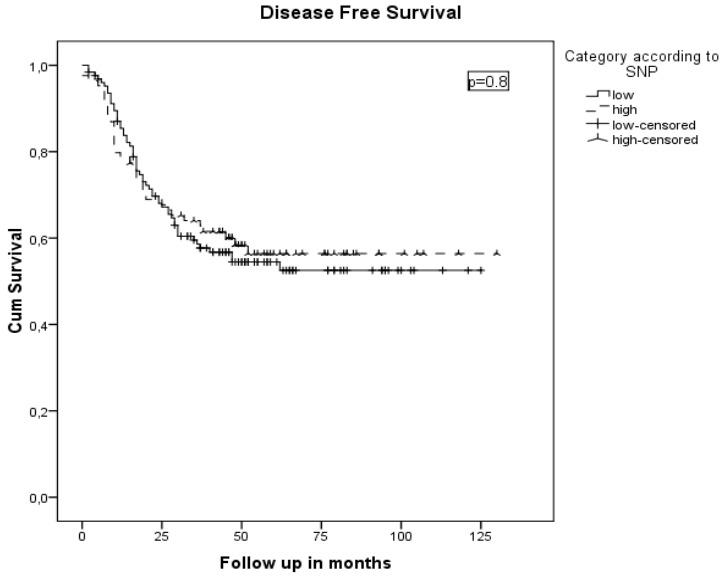
Analysis of the total population revealed no associations between the genotypes, either independently or in categories (as low and high expression), and overall survival, disease free survival or cancer specific survival (figure 3a and 3b). These results were confirmed by a multivariable Cox proportional hazard model including the following variables; T stage, N stage, differentiation grade, sex, tumor location and *TS* SNP category or VNTR category.

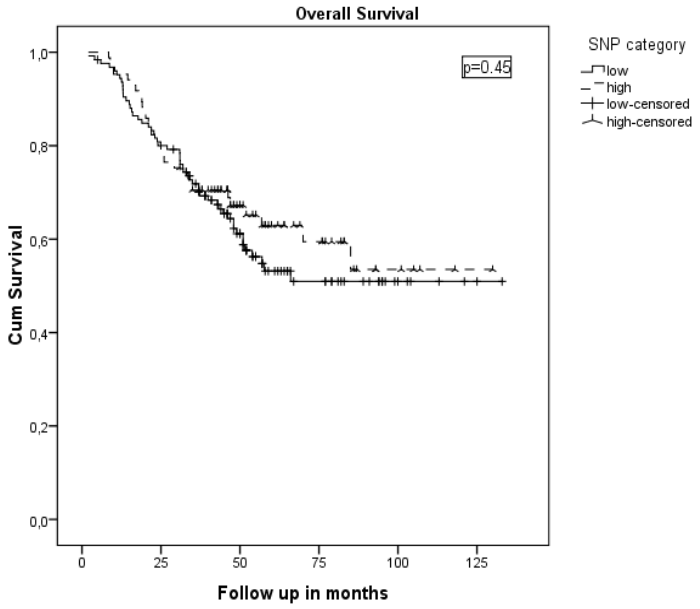
Mean time to progression in the groups according to SNP and VNTR category did not differ significantly (SNP category low 42 months and high 44 months. VNTR category low 42 months and high 42 months).

Since there was an association between *TYMS* gene polymorphisms and age, we stratified to age tertiles to study the effect of the polymorphism on survival in relation to age. As shown in figure 4, there was a difference between old and young patients. Moreover, this difference could be seen when we classified the patients as putative low and putative high TS expression according to the SNP (figure 4a) and to the VNTR alone (figure 4b). There was a switch in the genotype associated to a longer overall survival as the patients age increased. In other words, among patients younger than 60 years, the 2R homozygous had a better overall survival (p=0.02) whereas between patients older than 67 years, the ones with putative high TS expression (G allele) had a longer overall survival (p=0.06). These age dependent relations were also seen for CSS albeit not significant. However, there was no age dependent effect for DFS.

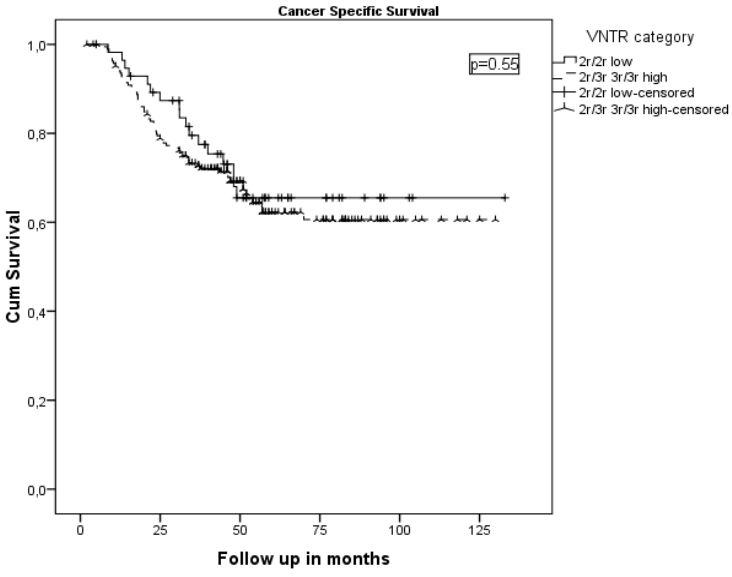
a)


Cancer Specific Survival
p=0.9
SNP category
low
high
low-censored
high-censored
Cum Survival
1,0
0,8
0,6
0,4
0,2
0,0
0
25
50
75
100
125
Follow up in months


Disease Free Survival
p=0.8
Category according to SNP
low
high
low-censored
high-censored
Cum Survival
1,0
0,8
0,6
0,4
0,2
0,0
0
25
50
75
100
125
Follow up in months


Overall Survival
p=0.45
SNP category
low
high
low-censored
high-censored
Cum Survival
1,0
0,8
0,6
0,4
0,2
0,0
0
25
50
75
100
125
Follow up in months

b)


Cancer Specific Survival
p=0.55
VNTR category
2r/2r low
2r/3r 3r/3r high
2r/2r low-censored
2r/3r 3r/3r high-censored
Cum Survival
1,0
0,8
0,6
0,4
0,2
0,0
0
25
50
75
100
125
Follow up in months

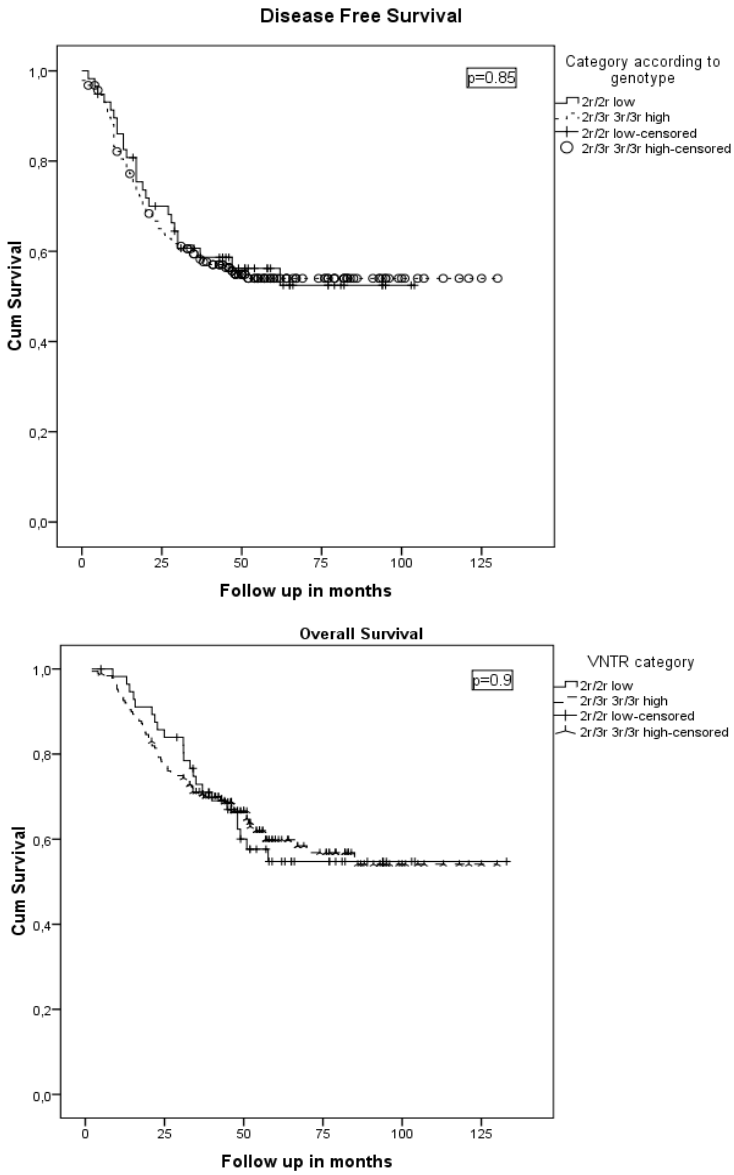
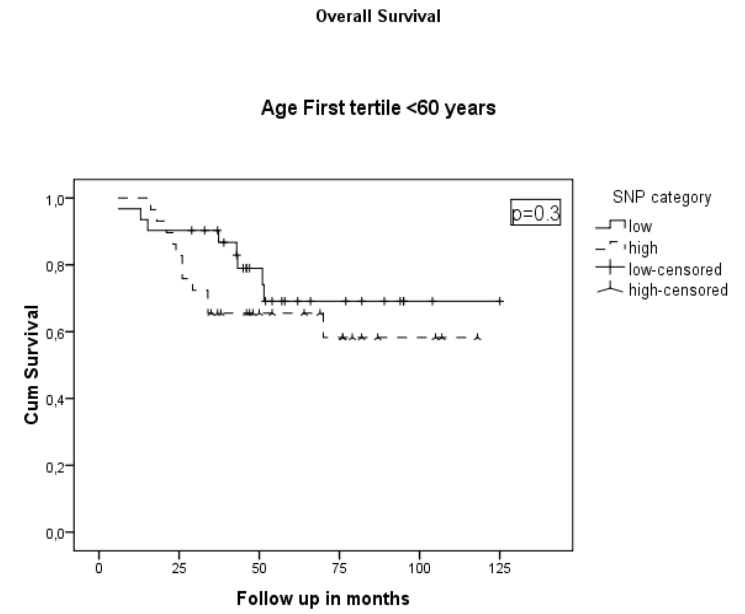
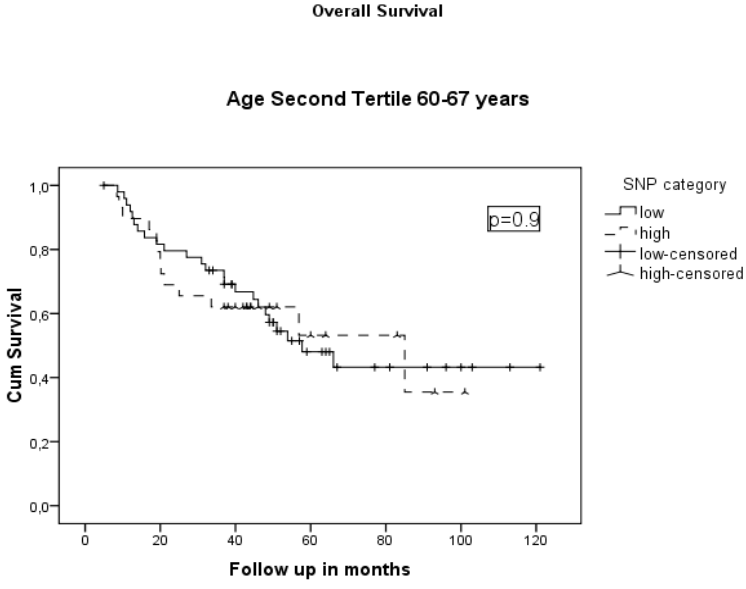
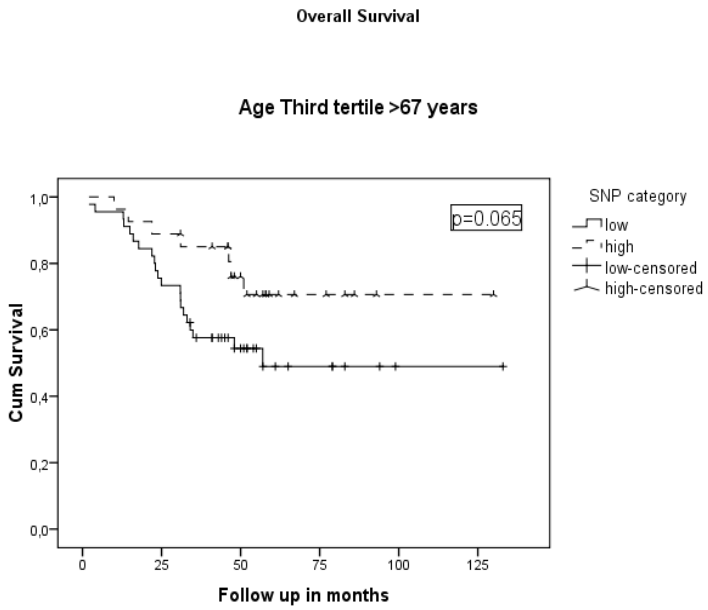


Figure 3: Kaplan Meier plots for OS, DFS, and CSS according to:
a) SNP categories TS low (2R homozygous plus C allele) and high (G allele) producers
b) VNTR categories (2R/2R low vs. 2R/3R and 3R/3R high).

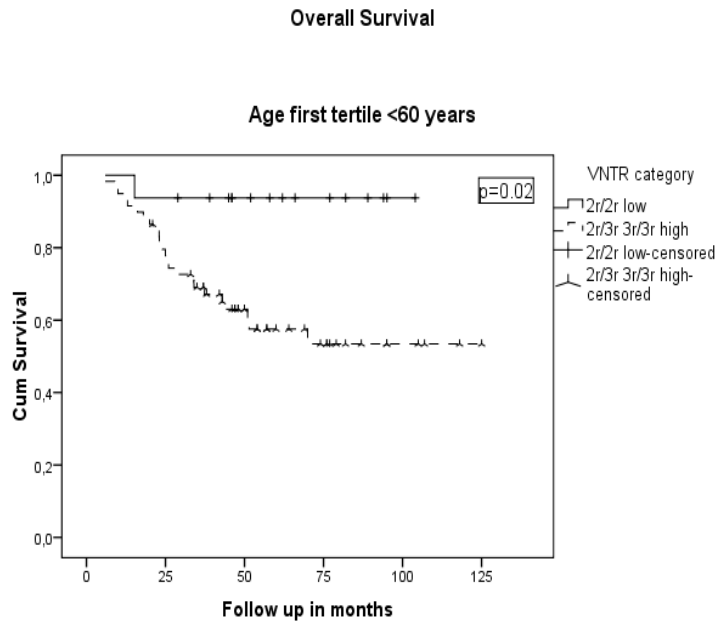
a)


Overall Survival
Age First tertile <60 years
p=0.3
SNP category
low
high
low-censored
high-censored
Cum Survival
1,0
0,8
0,6
0,4
0,2
0,0
0
25
50
75
100
125
Follow up in months


Overall Survival
Age Second Tertile 60-67 years
p=0.9
SNP category
low
high
low-censored
high-censored
Cum Survival
1,0
0,8
0,6
0,4
0,2
0,0
0
20
40
60
80
100
120
Follow up in months


Overall Survival
Age Third tertile >67 years
p=0.065
SNP category
low
high
low-censored
high-censored
Cum Survival
1,0
0,8
0,6
0,4
0,2
0,0
0
25
50
75
100
125
Follow up in months

b)


Overall Survival
Age first tertile <60 years
p=0.02
VNTR category
2r/2r low
2r/3r 3r/3r high
2r/2r low-censored
2r/3r 3r/3r high-censored
Cum Survival
1,0
0,8
0,6
0,4
0,2
0,0
0
25
50
75
100
125
Follow up in months

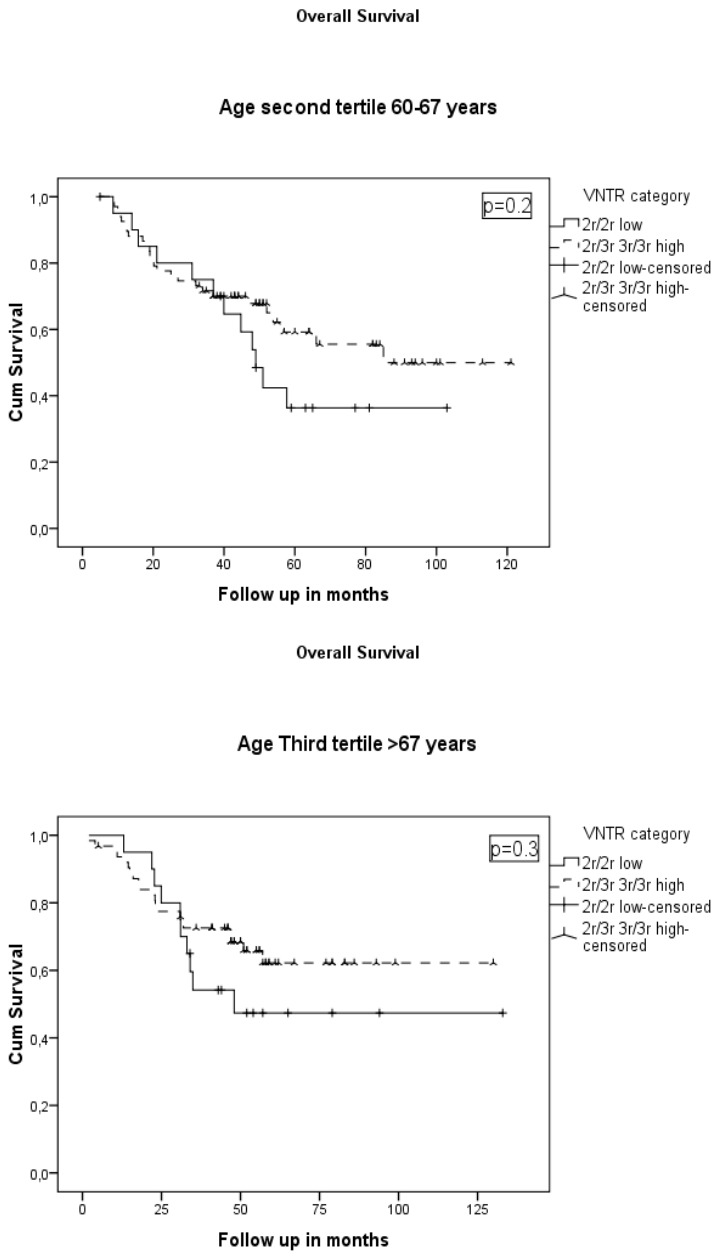


Figure 4: Kaplan Meier curves of the effects on overall survival of the VNTR and SNP categories stratified to age tertiles. a) SNP category b) VNTR category.

Discussion

Although, several studies have been published about the value of TS in colorectal cancers, as reviewed by Popat [40], the results are often contradictory and inconclusive particularly in patients treated adjuvantly.

Therefore this study aimed to elucidate the value of *TYMS* gene polymorphisms as possible molecular marker of therapy response in stage III colon carcinoma patients treated with adjuvant 5-FU chemotherapy.

In our well defined population of stage III colon cancer patients, *TYMS* genotype as determined by the SNP and the VNTR on the 5'UTR of the gene had no effect on patient outcome. There were no differences in survival (OS, DFS, CSS) between patients according to the genotypes independently or categorized as high en low TS expression based on either the *TYMS* SNP or on the VNTR alone (figure 3a and 3b). Although, in a previous publication we reported a predictive value for the *TYMS* VNTR [41], only a small number of patients were studied at that time and the apparently contradictory results could be explained by the difference in patient numbers between studies. In the present larger cohort of patients, we were not able to reproduce our previous results.

Moreover, inconsistent results over the predictive value of *TYMS* genotype and phenotype are a common feature in the literature. To our knowledge, there are at least, seven reports studying the value of both 5'UTR polymorphisms, VNTR and SNP, in colorectal carcinoma [6,31-33,35,44,45]. Our study agrees with Lecomte *et al*, Ruzzo *et al* and Prall *et al*; partly with Fernandez Contreras *et al* and argues with Kawakami *et al*, Marcuello *et al* and Lurje *et al*. In contrast with our patient population which consisted in stage III colon carcinoma patients only, all the previous publications included rectal carcinomas and studied either advanced colorectal cancer [33,35] or combined different disease stages [6,32,45]. We excluded rectal cancer patients because their treatment differs greatly from that of colon cancer patients. Rectal and colon cancer are likely to be two different diseases arising from different pathogenetic pathways and with different clinical behaviours [46]. We, as Prall *et al* included only stage III patients in order to have a homogeneous population. Accordingly, similar results were found although Prall and co workers included rectal cancer patients, albeit not neoadjuvantly treated and their patients' population was smaller [44].

Several authors have described a functional role of the *TYMS* 5'UTR polymorphisms on TS protein expression. 3R allele and G allele carriers would have a higher TS protein

level than homozygous 2R/2R or C allele carriers [30,31,34,42,43]. A higher TS expression has been described as a mechanism of 5-FU resistance[2], hence one would expect that carriers of the 3R allele and of the G allele would respond worse to 5-FU and have a poorer survival. Our results do not support this thesis. However, the regulation of TS expression and function remains quite complex and most likely is influenced by many still unknown factors [2,47]. Thus, ideally to explain the biological role of TS in the resistance to 5-FU, other techniques to objectively study protein expression and preferably function, would probably be more accurate. Therefore, our results based on DNA genotyping should not be interpreted as a biological explanation of 5-FU resistance mechanisms but as an answer to whether genotyping is a good marker for therapy response in colon cancer patients.

Interestingly, in our population, age seems to play a role on the *TYMS* genotype distribution and appears to modify the effects of the genotypes on survival. Indeed, the allelic distribution of both polymorphisms varied depending on age: the median age of the 3RC/3RG genotype was significantly lower in comparison to other genotypes. Similar results have been already reported by Odin *et al*. The authors described an inverse correlation between *TYMS* gene expression and age in colon cancer patients [48]. This relationship could point to a role of the *TYMS* gene polymorphisms in colon cancer risk. Hubner *et al* described a decreased risk of colon carcinoma between homozygotes for the *TYMS* 1491del6 on the 3'UTR of the gene. However, these authors did not find any role for the polymorphisms on the 5' UTR [49]. Further research is needed to study the allelic distribution in the normal population and to see whether this link remains significant.

Furthermore, the effect of the *TYMS* genotypes on overall survival was also modified by age. There was a switch in the *TYMS* genotypes associated to longer overall survival as age increased. In other words, genotypes associated with low TS expression (homozygous 2R and the carriers of the C allele) had a significant positive effect on survival among patients in the first age tertile category (corresponding to patients younger than sixty years). Conversely, these genotypes had a negative effect on survival among patients in the third age tertile category (i.e. older than sixty seven years). In the literature, an inverse association between *TYMS* gene expression, and age in colon cancer patients was already described by Odin *et al,* but the authors did not report its impact on survival.

To elucidate the underlying reasons of this age-dependent relation exceeds the scope

of this paper and needs additional research. Nevertheless, there is increasing evidence that age affects normal colonic mucosa and tumors. For instance, DNA methylation has been shown to increase with age in normal colonic mucosa [50,51] and different protein expression patterns have been found in the colonic mucosa of the elderly compared to that of younger people [52]. Moreover, Morris *et al* have also shown that the molecular aberrations in tumors differ according to age [53]. Thus, our finding supports the hypothesis that age probably modifies the effects of different molecular pathways on oncogenesis and on cancer progression.

In conclusion, the *TYMS* polymorphisms in the 5'UTR are not good markers of 5-FU therapy response in this population of stage III colon carcinoma patients. However, further research is necessary to study the role of age as an effect modifier of the polymorphisms on survival.

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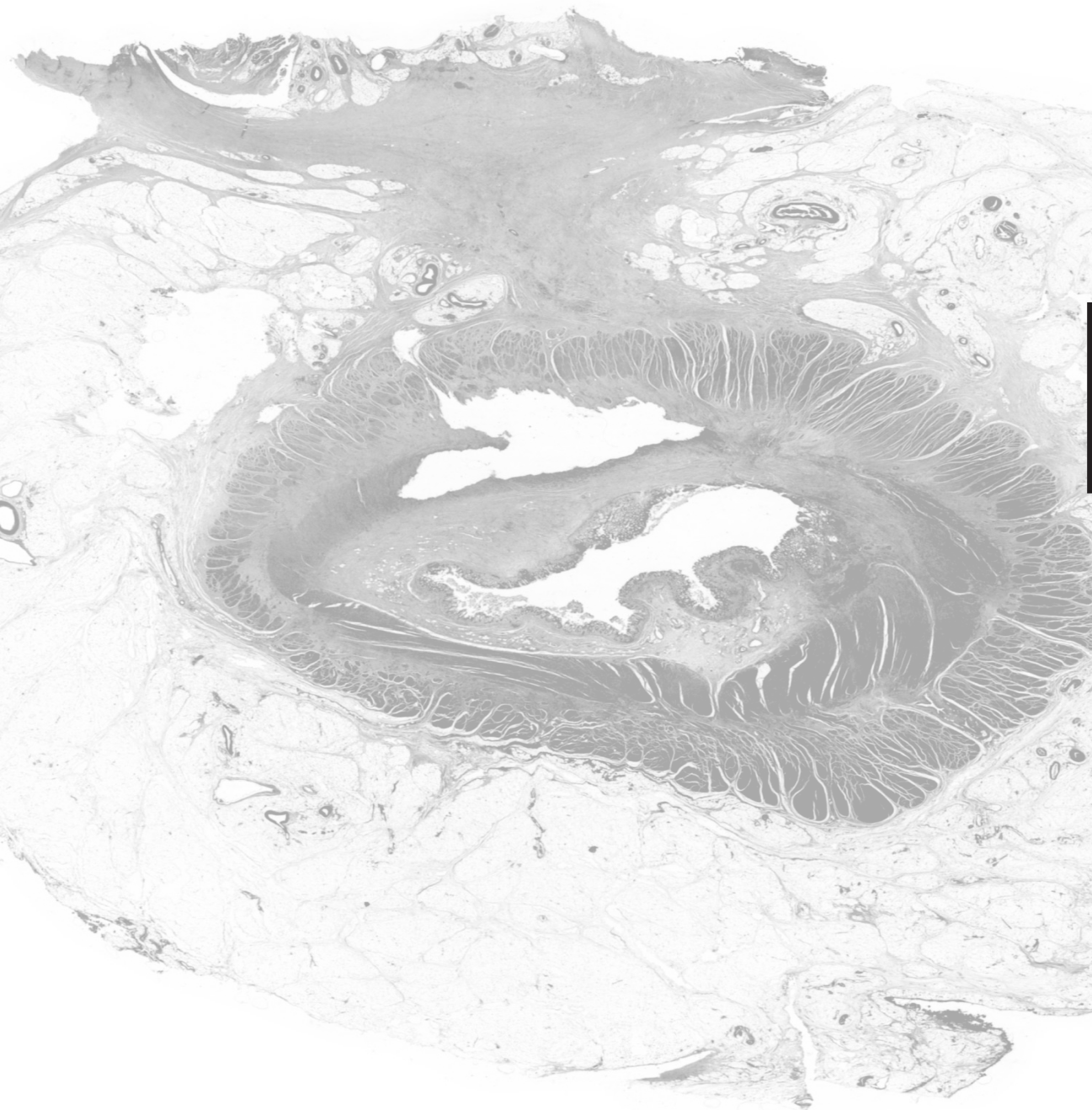

3

Value of gene polymorphisms as markers of 5-FU therapy response in stage III colon carcinoma: a pilot study

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A.J.C. van den Brule

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Abstract

The role of pharmacogenetics in chemotherapy response in colon carcinoma is controversial. We studied the value of known SNPs in genes involved in 5-FU metabolism as biomarkers of chemotherapy response in stage III colon carcinoma patients.

DNA was isolated from normal colonic tissue of 60 stage III colon carcinoma patients treated adjuvantly with 5-FU combined with leucovorin. The tested SNPs were validated SNPs on the *OPRT*, *TYMS* and *DPYD* genes and a synonymous SNP on the *TYMP* gene. Real time PCR, sequencing and RFLP were used for genotyping.

None of the studied genotypes was associated with any of the tumor or patient characteristics. Moreover, none of the genotypes studied had effect on patient survival. In conclusion, the tested SNPs are not biomarkers of chemotherapy response in our stage III colon cancer patients group.

Introduction

In colon cancer, the role of pharmacogenetics for drug toxicity and efficacy is still under discussion[1].

5-Fluorouracil (5-FU) is the main drug of choice in the treatment of stage III colon carcinoma. Several proteins are involved in the metabolism of 5-FU and many of the genes coding for these proteins have been shown to be polymorphic.

Ororate phosphorybosil transferase (OPRT) and thymidine phosphorylase (TP) activate 5-FU by phosphorylation into active metabolites which respectively incorporate into RNA or inhibit thymidylate synthase (TS). Dihydropyrimidine dehydrogenase (DPD) inactivates 5-FU in the liver [2,3]. The genes encoding for these proteins harbor functional polymorphisms.

The *OPRT* gene contains several polymorphisms, among those the G638C SNP that causes a glycine by alanine substitution at position 213 of the protein, which has been associated with a higher expression and activity of the protein and with an increased toxicity of 5-FU therapy [4].

There are several polymorphisms described in the TP gene (*TYMP)*, however there are no confirmed polymorphisms in coding regions causing changes in aminoacid sequence. The value of these polymorphisms as markers of response to 5-FU therapy is to our knowledge, unknown.

The polymorphisms in the enhancer region of the TS gene (*TYMS*) have been widely studied in their relation to response to 5-FU therapy and with protein expression and activity. The studied polymorphisms consist in a 28bp repeat at the 5' untranslated region of the gene and a G>C SNP in the second repeat of the three repeat allele. In the Caucasian population the variants with two (2R) or three (3R) repeats are the most frequent alleles found. On the basis of the effects of the SNP in the second repeat of the 3R allele on TS protein expression, patients could be classified as high TS protein producers when carrying the G allele and low TS protein producers when carrying the C allele [5,6]. However, up to date, the results concerning the effect of these polymorphisms in 5-FU response remain inconclusive [7].

Finally, the *DPYD* gene has been shown to play a very important role in toxicity of 5-FU. The polymorphism in the exon/intron boundary at exon 14 is responsible for severe toxicity in these patients [8]. However, little is known about the value of these and other polymorphisms as markers of response.

We aimed to study the value of known polymorphisms in the *OPRT, TYMP, TYMS* and *DPYD* genes as markers of response in stage III colon carcinoma patients treated with 5-FU chemotherapy in combination with leucovorin.

Materials and methods

Patient material

Sixty stage III colon carcinoma patients treated with surgery and 5-FU chemotherapy following the Mayo regime were studied.
All diagnoses were made at a central laboratory for pathology between 2003 and 2004. Population data were obtained from the cancer registry database of the Comprehensive Cancer Centre South. Follow-up information was obtained from medical records.
The use of clinical material for this retrospective study was approved by the institutional review board according to the guidelines of the Dutch Federation of Research Associations.
DNA was isolated from normal colonic mucosa from formalin fixed paraffin embedded (FFPE) material after proteinase K digestion and purification using the HPTTP kit (Roche, Almere, the Netherlands).

Target genes and polymorphisms

Polymorphisms must be non synonymous and confirmed by independent research groups. However, in the case of the *TYMP* gene, there were no confirmed non synonymous polymorphisms and therefore we chose one confirmed synonymous polymorphism.

OPRT

The G638C SNP (rs1801019) causing a Gly213Ala substitution was tested by real time PCR with the following primers and probes forward 5' GCT GAG ACA GTT GGG AGA GTG A 3', reverse 5' TGA GTT CTT TGG GTG CTT CCT T 3', probe for G allele 6FAM 5'CGA ATC ATA ATG GTT C3'and probe for C allele 6FAM 5'AGC GAA TCA TAA TGC TT3'. Reactions were performed using Roche chemistry in a final volume of 20 µl in the light cycler v2 (Roche, Almere, the Netherlands).

TYMP

The rs470119 SNP was assessed by restriction fragment length polymorphism (RFLP). PCR was performed using the following primers forward 6FAM-5'TCC AGA GCC CAG GTA3' and reverse 5'CTG GCC AGG GTC TCC ATC A3'. The 71 bp long PCR product was then digested with MboI restriction enzyme (New England Biolabs, Ipswich, United

Kingdom). After digestion, fragment length analysis was carried out by capillary electrophoresis. The following fragment length was expected for homozygous GG, 40bp and 30bp, for AA 71 bp and for heterozygous AG 71bp, 40bp and 30bp.

TYMS

The two polymorphisms in the enhancer region of this gene were typed using PCR and RFLP as described elsewhere 9. Briefly, the 28 bp repeat was typed by PCR means followed by electrophoresis on 2% agarose gel. The G>C SNP was typed by digestion of the PCR product with HaeIII restriction enzyme. The G to C substitution abrogates the restriction site for this enzyme. Subsequently, products were separated by agarose gel electrophoresis.

DYPD

The SNP A1627G (rs1801159) causing the substitution of isoleucine by valine at position 543 of the protein was tested by PCR followed by sequencing using the following primers, forward 5'GCA GTC ACA ATA TGG AGC3' and reverse 5'TTA CCT TAT CAA GAG AGA AAG TT3'. The expected length of the product was 225 bp. Subsequently, PCR products were purified using enzymatic purification with ExoSapIT (USB, USA) and the sequencing reaction was performed using the same primers as for the PCR reaction and Big Dye chemistry (Applied Biosystems, Nieuwerkerk aan den Ijssel, the Netherlands). Sequences were analyzed using the sequencing analysis 5.3.3 software (Applied Biosystems)

Statistical Analysis

SPSS v.16 software package for Windows (Chicago, Il., U.S.A.) was used for statistical analysis. Categorical data were analyzed by means of a chi-square or Fischer's exact test. The end point of this study was progression free survival (PFS defined as time between surgery and disease progression). Univariate survival analysis was performed by Kaplan Meier analysis and differences were analyzed using the Log Rank method. Hazard Ratios and multivariate analysis were calculated using the Cox Proportional Hazard model.

All tests are two tailed and a result was considered significant when $p<=0.05$.

Results

Briefly, characteristics of the sixty patients studied were as follows, median age at diagnosis was 64 years (range 30-81), fifty two percent (n=31) of the tumors were located on the right side of the colon and 53% (n=32) of the patients were male. The majority 70% (n=42) had a T3 tumor. Median follow up was 39 months (range 2-57). 40% was still alive without evidence of disease at the end of the follow up, 24% had developed a local recidive or a distant metastasis, 31% was dead because of cancer related causes and 5.2% was dead because of non cancer related causes as specified in their medical records. Median time to progression was 15 months (range 6-47)

Frequencies of the different alleles are shown in table 1. All frequencies followed Hardy-Weinberg equilibrium and did not differ significantly from frequencies published on the HapMap database for the Caucasian population.

In this group of patients, there were no significant associations between any of the genotypes found and any of the clinical and histopathological variables tested including gender, tumor location, T stage and N stage.

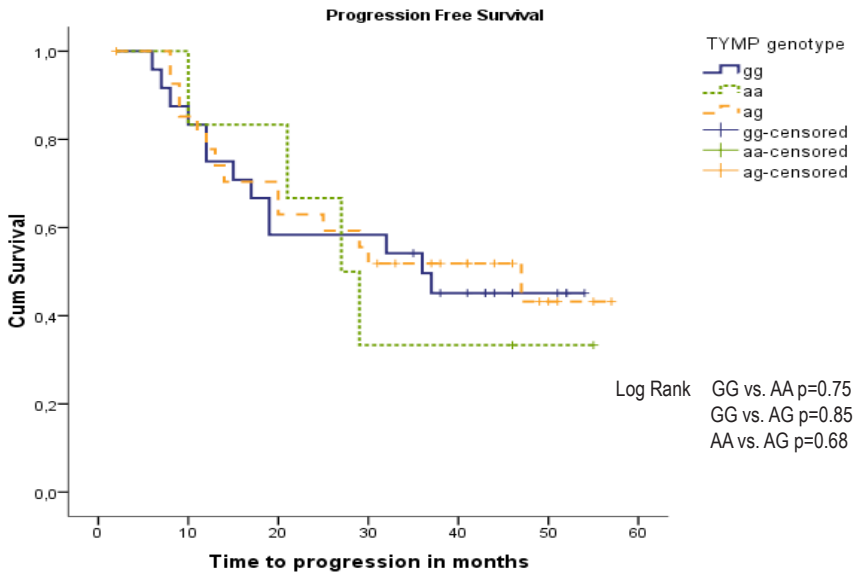
Survival Analysis

For the survival analysis, TYMS SNP genotypes were grouped as putative high TS expression (genotypes 2R/3G, 3C/3G and 3G/3G) and putative low TS expression (2R/2R, 2R/3C. 3C/3C). No effect on progression free survival of the several genotypes was seen in a univariate (figure 1) or in a multivariate survival analysis, containing other known prognostic variables for colon carcinoma such as T stage and N stage of the tumor.

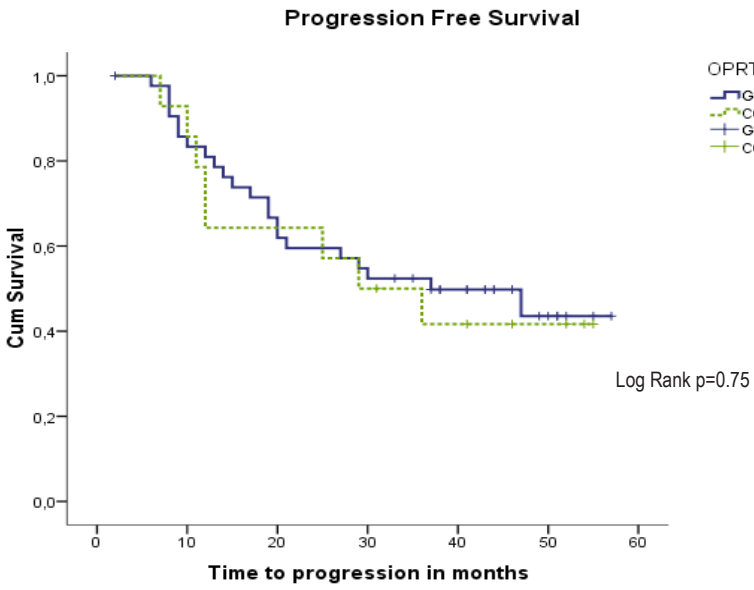
Table 1: Allelic frequencies for each SNP.

SNPs	N (%)
***OPRT* G638C**	
GG	44 (75)
GC	15 (25)
CC	0 (0)
***TYMP* G6601A**	
GG	25 (42)
AG	29 (48)
AA	6 (10)
***TYMS* VNTR28bp**	
2 repeats	10 (17)
2 & 3 repeats	35 (58)
3 repeats	15 (25)
***TYMS* SNP**	
2R/2R	10 (16.7)
2R/3RC	16 (26.7)
2R/3RG	19 (31.7)
3RC/3RC	7 (11.7)
3RG/3RC	4 (6.7)
3RG/3RG	4 (6.7)
***DPYD* A1627G**	
AA	37 (67)
AG	14 (26)
GG	4 (7)

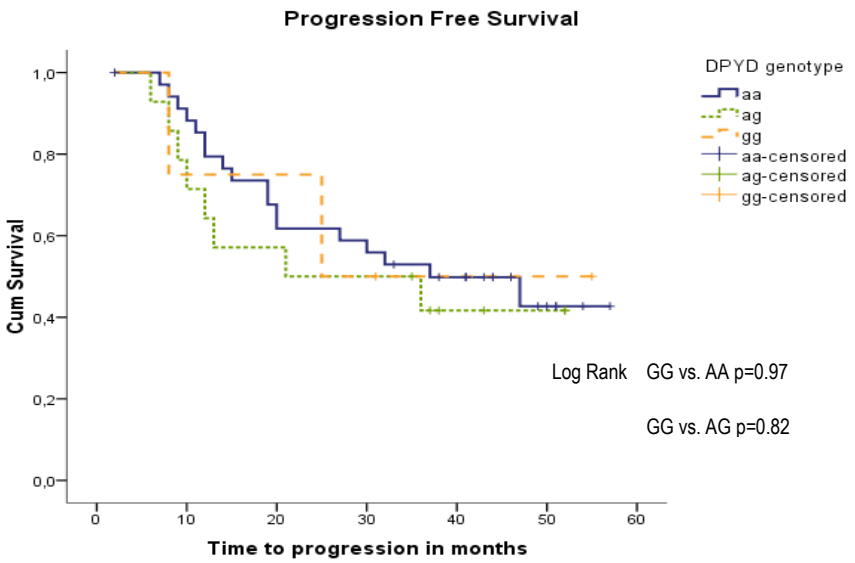
a)


Progression Free Survival
Cum Survival
1,0
0,8
0,6
0,4
0,2
0,0
0
10
20
30
40
50
60
Time to progression in months
TYMP genotype
gg
aa
ag
gg-censored
aa-censored
ag-censored
Log Rank GG vs. AA p=0.75
GG vs. AG p=0.85
AA vs. AG p=0.68

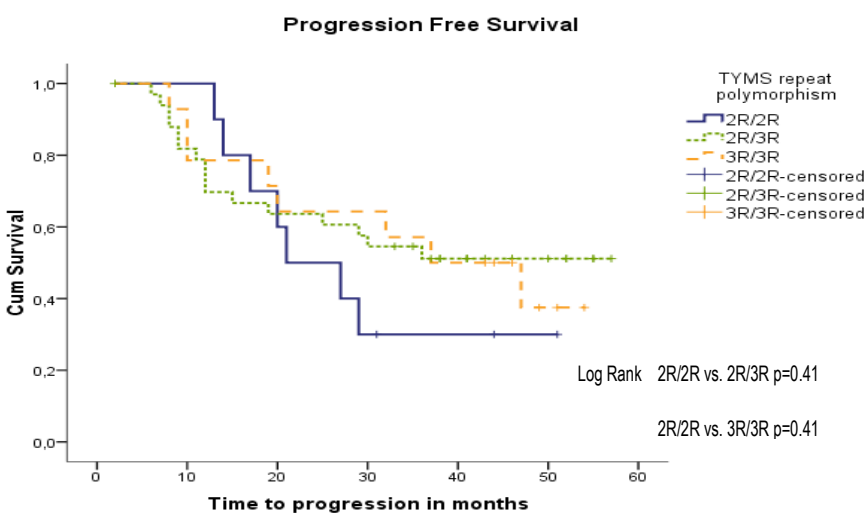
b)


Progression Free Survival
Cum Survival
1,0
0,8
0,6
0,4
0,2
0,0
0
10
20
30
40
50
60
Time to progression in months
OPRT genotype
GG
CG
GG-censored
CG-censored
Log Rank p=0.75

c)


Progression Free Survival
Cum Survival
1,0
0,8
0,6
0,4
0,2
0,0
0
10
20
30
40
50
60
Time to progression in months
DPYD genotype
aa
ag
gg
aa-censored
ag-censored
gg-censored
Log Rank GG vs. AA p=0.97
GG vs. AG p=0.82

d)


Progression Free Survival
Cum Survival
1,0
0,8
0,6
0,4
0,2
0,0
0
10
20
30
40
50
60
Time to progression in months
TYMS repeat polymorphism
2R/2R
2R/3R
3R/3R
2R/2R-censored
2R/3R-censored
3R/3R-censored
Log Rank 2R/2R vs. 2R/3R p=0.41
2R/2R vs. 3R/3R p=0.41

e)

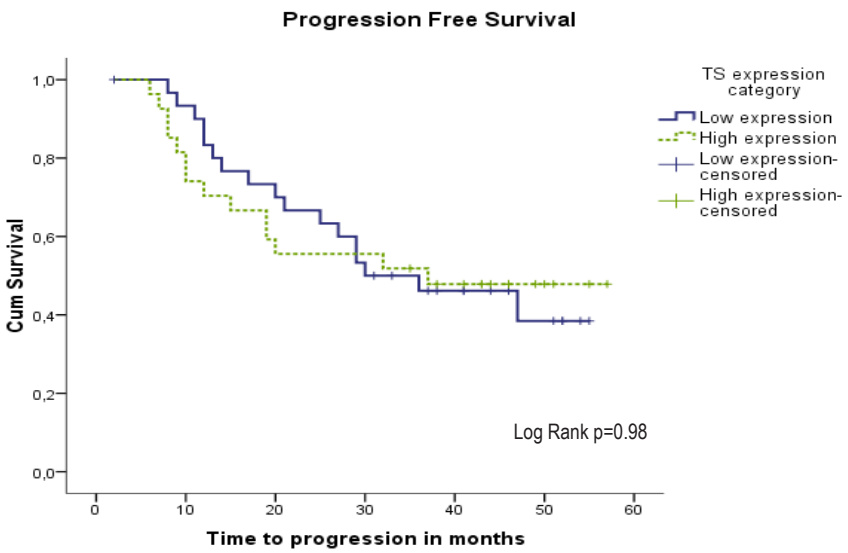


Figure 1: Kaplan Meier plots for PFS according to all genotypes tested; a) *TYMP* G6601A genotypes, b) *OPRT* G638C, c) *DPYD* A1627G, d)*TYMS* 28bp VNTR, e) TYMS expression category determined by SNP genotypes (Low expression 2R/2R, 2R/3C, 3C/3C; High expression 2R/3G and 3G/3G).

Discussion

We aimed to study whether known SNPs in genes involved in 5-FU metabolism were good markers of therapy response in stage III colon carcinoma patients. According to our results, we conclude that the SNPs tested in the *OPRT, TYMP, TYMS* and *DPYD* genes are not good markers of therapy response in the present cohort. No effect on survival of the different genotypes was seen, however, results should be considered with caution due to the small number of patients analyzed.

Although, increased expression of *OPRT* mRNA and protein activity have been related to a shorter survival of colorectal carcinoma patients treated with 5-FU [10-12] together with the fact that the studied SNP has been proven to be associated to a higher protein expression and activity [13 4], no effects of the different genotypes in DFS was seen in the present cohort. The *OPRT* CC variant is very rare in the general population. Our results reflect the low frequency of this genotype in the Caucasian population. If the CC variant would have an effect on survival, the fact that it is such a rare variant makes it very difficult to prove since numbers of patients needed would be very large.

The existing literature is more conclusive about TP protein expression which seems to have no influence in survival of colorectal patients [14-19]. Accordingly, the SNP in the *TYMP* gene was not a good marker for therapy response in our group.

The role of the typed polymorphisms on the *TYMS* gene remains controversial. It has been widely studied as protein and mRNA expression as well as DNA genotyping, still the results are inconclusive as reviewed by Popat *et al* [7]. These contradictory results are probably due to differences in methodology, technology and patients' selection. In the present patient group, *TYMS* polymorphisms in the enhancer region are not good markers of therapy response, even when grouping patient by category of putative TS expression no difference in survival was found between putative low and high producers. These results agree with previous results in a larger cohort of patients [20].

Finally, although *DPYD* has been widely studied in relation to 5-FU toxicity, it also seemed to have a role in outcome [15,19,21-23]. However, whether this effect is due to the enzyme itself or due to high toxicity and subsequent therapy interruption is not clear. In the present cohort of patients, the studied SNP is not a good marker of therapy response.

In conclusion, polymorphisms in genes involved in 5-FU metabolism are not valuable as markers of response in the present cohort of colon cancer patients. Recently, the

results of a clinical trial over the value of some polymorphisms in predicting toxicity in a large group of colon carcinoma patients failed to find any relation between the tested SNPs and toxicity [1]. In that study the value of several SNPs involved in 5-FU metabolism was tested in relation to toxicity. In the same way, we cannot show any value of these SNPs as markers of response to 5-FU in our group of patients.

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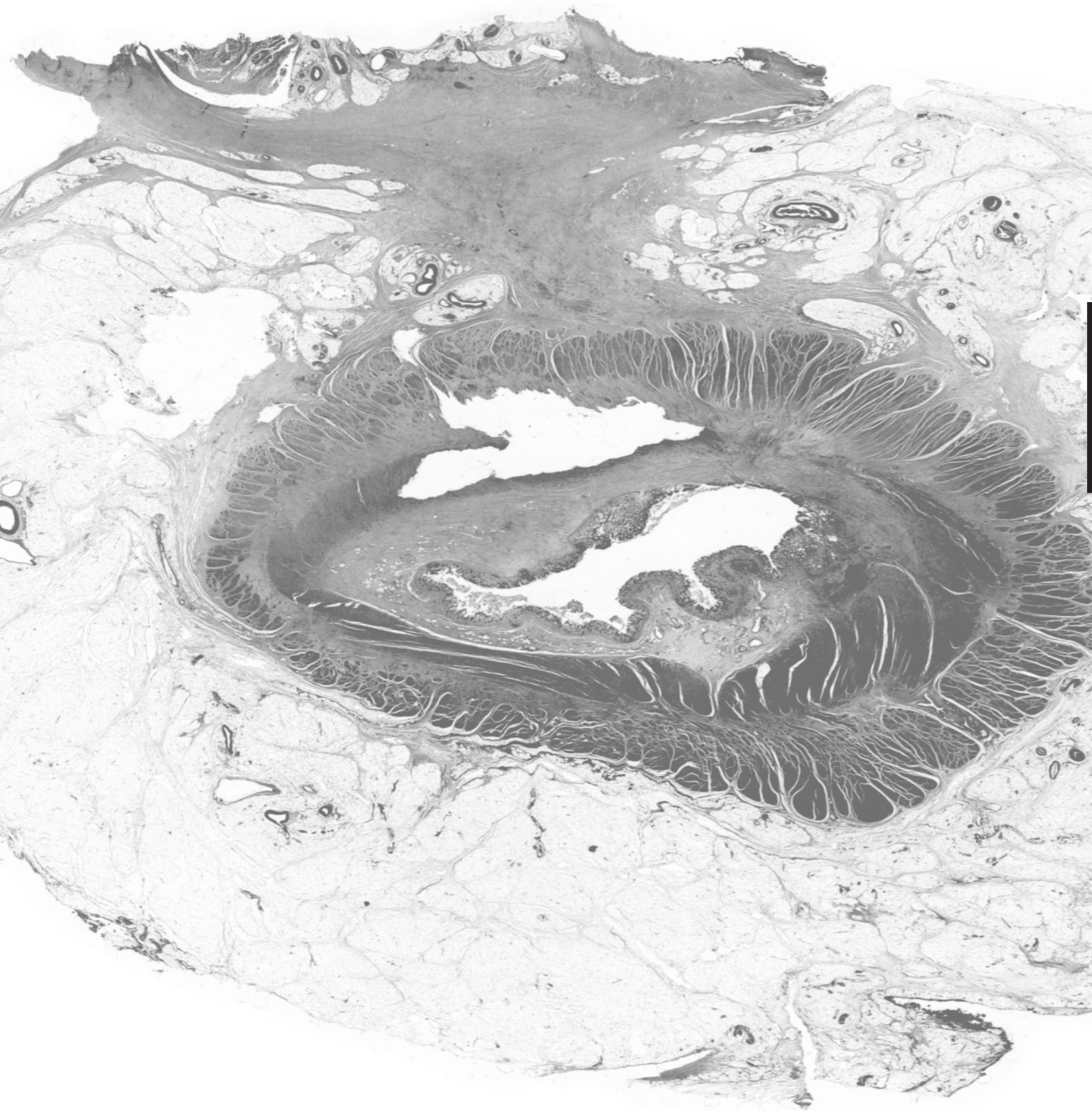

4

Pharmacogenetics of oxaliplatin as adjuvant treatment in colon carcinoma: Are single nucleotide polymorphisms in *GSTP1*, *ERCC1* and *ERCC2* good predictive markers?

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Abstract

Adjuvant chemotherapy improves survival in stage III colon cancer patients. However, a subgroup of patients still develops recurrent disease at some point in time, partly because of the ineffectiveness of the chemotherapy. Predictive markers of response are therefore crucial. Our aim was to study the predictive value of functional polymorphisms in genes involved in the metabolism of oxaliplatin and in DNA repair in stage III colon cancer patients.

Normal DNA was isolated from 98 patients diagnosed with stage III colon carcinoma. Single nucleotide polymorphisms (SNPs) in three genes (the excision repair cross-complementing genes *ERCC1* 19007T>C and *ERCC2* 2251A>C, and the glutathione S-transferase pi 1 gene *GSTPI* 313A>G were tested by PCR followed by digestion with restriction enzymes or by direct sequencing. These genes and SNPs were selected on the basis of their reported associations with oxaliplatin response in colorectal cancer.

The genotype frequencies were in Hardy-Weinberg equilibrium. *GSTPI* and *ERCC2* polymorphisms were significantly associated with sex. The AA genotype of *GSTPI* 313A>G was more frequent in men than in women (59% vs 30%, p = 0.02). The CC genotype of *ERCC2* 2251A>C was significantly more frequent in women than in men (24% vs 6%, p = 0.02). In univariate and multivariate survival analysis, none of the tested polymorphisms seemed to influence disease-free survival. The *GSTPI* AA genotype had different effects on survival between men and women; homozygous A men had significantly worse cancer-specific survival and overall survival than women with the same genotype (log rank p = 0.029 and p = 0.015, respectively).

None of the tested polymorphisms is likely to be a reliable marker of response to oxaliplatin therapy. The *GSTPI* 313A>G homozygous A genotype may have a prognostic value in male patients.

Introduction

Oxaliplatin was approved for adjuvant treatment of stage III colon carcinoma patients in the Netherlands in 2004. When administered in combination with fluorouracil, this platinum compound has been proven to decrease the recurrence risk by 23% in the first 3 years after surgery and to increase overall survival by 4.2% after 6 years of follow-up[1-3]. Age and the presence of comorbidity are known factors limiting the use of chemotherapy, even when chemotherapy is advised according to guidelines[4,5]. In this regard, patients with stage III disease form a very interesting study group, since chemotherapy is given in an adjuvant setting to increase survival by decreasing recurrence rates. Thus, in stage III disease, chemotherapy has the potential to be curative. Within this disease stage, markers of response are essential to increase therapy success rates and decrease toxicity due to unnecessary exposure to drugs.

The mechanism of action of platinum compounds is through the generation of DNA platinum adducts, leading to intrastrand crosslinks. Thereafter, DNA synthesis will be inhibited and the cell will undergo apoptosis[3]. Two main mechanisms are involved in oxaliplatin resistance: on one hand there is increased intracellular detoxification, mediated by glutathione-S-transferase proteins; and on the other hand there is increased activity of the nucleotide excision repair system, which is involved in repairing DNA damage specifically caused by oxaliplatin[6,7].

Several polymorphisms in the genes coding for detoxification enzymes (glutathione S-transferase pi 1 [*GSTPI*], glutathione S-transferase theta 1 [*GSTT1*], and glutathione S-transferase mu 1 [*GSTM1*]) and the nucleotide excision repair system (excision repair cross-complementing rodent repair deficiency, complementation group 2 [*ERCC2*], excision repair cross-complementing rodent repair deficiency, complementation group 1 [*ERCC1*], X-ray repair complementing defective repair in Chinese hamster cells 1 [*XRCC1*], and xeroderma pigmentosum, complementation group A [*XPA*]) have been studied in the context of resistance to platinum compounds[8,9] in different types of cancer such as colon carcinoma, head and neck tumors[10], esophageal cancer[11], and lung cancer[12]. The use of different study designs, control groups, cancer types, and therapies makes the study results difficult to interpret and often discordant.

In the case of colon carcinoma, the single nucleotide polymorphisms (SNPs) *ERCC1* 19007T>C (Asn118Asn), *ERCC2* 2251A>C (Lys751Glyn), and *GSTP1* 313A>G (Ile105Val) have been frequently studied and associated with response to oxaliplatin, but mostly

in metastatic colorectal cancer where patients receive several lines of combination therapy[3,8,9,13-19].

However, findings on the effects of these SNPs on protein production – and, more importantly, on protein function – have been inconclusive. For the *GSTP1* 313A>G polymorphism only, a consensus has been reached regarding the functional consequences, i.e. the variant allele is associated with decreased conjugating activity[3]. The functional findings on *ERCC1* 19007T>C and *ERCC2* 2251A>C have been more controversial. Although *ERCC1* 19007T>C is a synonymous SNP which does not cause amino acid substitution, ERCC1 expression has been shown to be decreased in patients with the variant allele (19007C) [20]. The functional reports on the effects of the 2251A>C SNP in *ERCC2* have been contradictory. One study concluded that homozygous C would have less DNA repair capacity in the lung [12], whereas other researchers have reported suboptimal DNA repair capacity in homozygous A [21]. Despite the unclear functional role of these SNPs and the inconsistent results obtained in metastatic colon cancer patients, we aimed to determine the predictive value of the three SNPs – namely, *ERCC1* 19007T>C, *ERCC2* 2251A>C, and *GSTP1* 313A>G – in stage III colon cancer patients where oxaliplatin was administered as adjuvant therapy.

Materials and Methods

Patients

Patients (n = 98) with stage III colon carcinoma diagnosed at the Pathologic Anatomy and Medical Microbiology (PAMM) Laboratory (Eindhoven, the Netherlands) were included in this retrospective study. All patients were treated with curative intended surgery followed by adjuvant chemotherapy. Fifty-three patients (54%) followed either the FOLFOX regimen (leucovorin, bolus fluorouracil, and oxaliplatin) or the XELOX (oxaliplatin and oral capecitabine) regimen, whereas 45 patients (46%) followed the Mayo regimen (leucovorin and bolus fluorouracil). The median age and sex distribution did not differ significantly between these two groups. Demographic data were obtained from the cancer registry managed by the Comprehensive Cancer Centre South (Integraal Kankercentrum Zuid [IKZ]; Eindhoven, the Netherlands). Clinical data were obtained from the patients' medical records between April 2009 and January 2010. The median follow-up period was 37 months (range 2–57 months). The use of clinical material for this retrospective study was approved by the local institutional review board, according to the guidelines of the Dutch Federation of Biomedical Scientific Societies (FMWV), reviewed in January 2009.

DNA was isolated from formalin-fixed paraffin-embedded normal colonic mucosa, using an HPTPP kit (Roche, Almere, the Netherlands) after proteinase K digestion, according to the manufacturer's instructions.

Single Nucleotide Polymorphism Typing

ERCC1 19007T>C

This SNP, consisting of a T>C transition at codon 118 (rs11615) was typed by means of PCR and restriction fragment length polymorphism. PCR was performed using the following primers: forward 5'GCA GAG CTC ACC TGA GGA AC3' and reverse 5'GAG GTG CAA GAA GAG GTG GA3', as described elsewhere[8].Subsequently, the PCR products were digested with the *Bsr*DI restriction enzyme (New England Biolabs, Hitchin, UK), which digests the product into two fragments of 117 bp and 82 bp in length, respectively, if the T allele is present.

ERCC2 2251A>C

The *ERCC2* 2251A>C SNP (rs13181), causing the substitution of lysine with glutamine in codon 751, was determined by PCR followed by direct sequencing. To briefly summarize, PCR was performed using the following primers: forward 5'TGC CCC CCT CTC CCT TT 3' and reverse 5'CCA GGG CCA GGC AAG ACT 3'. The PCR products were subsequently purified using ExoSAP-IT (USB Europe GmbH, Staufen, Germany), according to the manufacturer's instructions. Thereafter, a sequencing reaction was performed using Applied Biosystems chemistry (Applied Biosystems, Nieuwerkerk aan den IJssel, the Netherlands). Finally, sequence products were analyzed on an Applied Biosystems ABI 3130, using sequencing analysis software from Applied Biosystems.

GSTP1 313A>G

The *GSTP1* polymorphism in codon 105 (rs1695), causing the substitution of isoleucine with valine, was typed by means of PCR and subsequent restriction enzyme digestion. First, PCR was performed with the following primers: forward 5'ACC CCA GGG CTC TAT GGG AA3' and reverse 5'TGA GGG CAC AAG AAG CCC CT3', as published elsewhere [8]. A single product of 176 bp in length was obtained, which was subsequently digested with the *Bsm*AI restriction enzyme (New England Biolabs). Two products of 88 bp each were obtained after digestion of the G allele, whereas the product remained undigested when the A allele was present.

Statistical Analysis

All statistical analyses were performed using the SPSS v.16 package for Windows (Chicago, IL, USA).

A χ^2 test and Fischer exact tests were performed to study associations between categorical variables. One-way analysis of variance was used to study associations between categorical and continuous variables.

To study the predictive value of the variables that were tested, we chose as primary end points disease-free survival, defined as the time between curative surgery and the development of either a distant metastasis or a local recurrence, and cancer-specific survival, defined as the period of time from curative surgery until cancer-related death. As a secondary end point, overall survival was defined as the time between curative surgery and death from any cause.

Univariate survival analysis was performed by Kaplan-Meier analysis, and differences

between curves were analyzed using the log-rank method. Hazard ratios and multivariate analysis were calculated using the Cox proportional hazard model. All tests were two-tailed, and the results were considered significant when $p \leq 0.05$.

Results

The patient characteristics, allelic frequencies, and associations between variables are presented in table 1. In summary, the median age was 64 years (range 30–85 years), 59 of 98 patients (60%) were male, 52 of 98 tumors (54%) were located on the right side of the colon (between the coecum and the splenic flexure), and 64 (65%) were classified as T3 tumors. The median follow-up period was 37 months (range 2–57 months). At the end of the follow-up period, 46 patients (47%) had no evidence of cancer, 21 (22%) were alive with cancer, 26 patients (27%) had died from cancer-related causes, and 4 patients (4%) had died from non-cancer-related causes, according to their medical records.

Because of the poor DNA quality in some samples or PCR inhibition, not all samples could be analysed for all three SNPs.

All genotypes that were studied were in Hardy-Weinberg equilibrium. The allelic frequencies of all three SNPs were in agreement with frequencies published for Caucasian populations on the website of the US National Institute for Biotechnology and Information (www.ncbi.nlm.nih.gov; reviewed in October 2010). A significant association was found between *ERCC2* 2251A>C and sex. The CC genotype was more frequent in women than in men (24% vs 6%, p = 0.02). Moreover, the *GSTP1* 313A>G SNP was also significantly associated with sex. Men were more frequently homozygous A than women (59% vs 30%, p = 0.02). No further statistically significant associations between the studied variables were found.

No significant effects of any of the tested polymorphisms on clinical outcomes were seen (figure 1 shows disease-free survival; cancer-specific survival and overall survival are not shown).

In the multivariate survival analysis, which included sex and age in the model, none of the SNPs were associated with a higher hazard ratio for disease-free survival, cancer-specific survival, or overall survival (table 2 shows disease-free survival).

Because of the significant association between *ERCC2* 2251A>C and *GSTP1* 313A>G and sex, we also analyzed the effects of these polymorphisms on survival according to sex. There was no significant interaction between the *ERCC2* 2251A>C polymorphism and sex in relation to disease outcome. Conversely, *GSTP1* 313A>G seemed to influence prognosis differently in men than in women; homozygosity A conferred significantly poorer cancer-specific survival and overall survival in men than in women (log rank p =

0.03 for cancer-specific survival and p = 0.0015 for overall survival) as compared with the other genotypes (figure 2), but no such effect on disease-free survival was seen. This association was independent of the therapy received by the patients.

Table 1: Patient characteristics, genotype frequencies, and associations between variables.

Variable	N (%)	*ERCC1* 19007T>C			*ERCC2* 2251A>C			*GSTPI* 313A>G		
		TT	CT	CC	AA	AC	CC	AA	AG	GG
***ERCC1* 19007T>C**										
TT	34 (39)				10 (36)	13 (46)	5 (18)	17 (59)	9 (31)	3 (10)
CT	44 (50)				15 (40)	17(46)	5 (14)	13 (35)	21 (57)	3 (8)
CC	10 (11)				4 (57)	3 (43)	0 (0)	4 (44)	5 (56)	0 (0)
***ERCC2* 2251A>C**										
AA	32 (40)	10 (34)	15 (52)	4 (14)				17 (53)	13 (41)	2 (6)
AC	38 (47)	13 (39)	17 (52)	3 (9)				16 (43)	18 (49)	3 (8)
CC	10 (13)	5 (50)	5 (50)	0 (0)				5 (50)	3 (30)	2 (20)
***GSTPI* 313A>G**										
AA	46 (48)	17 (50)	13 (38)	4 (12)	17 (45)	16 (42)	5(13)			
AG	41 (43)	9 (26)	21(60)	5 (14)	13 (38)	18 (53)	3 (9)			
GG	9 (9)	3 (50)	3 (50)	0 (0)	2 (28.5)	3 (43)	2 (28.5)			
Adjuvant therapy										
fluorouracil + oxaliplatin	53 (54)	20 (40)	22 (44)	8 (16)	16 (36)	23 (51)	6 (13)	24 (46)	23 (44)	5 (10)
fluorouracil	45 (46)	14 (37)	22 (48)	2 (5)	16 (46)	15 (43)	4 (11)	22 (50)	18 (41)	4 (9)
Follow-up status										
no evidence of disease	46 (47)	18 (43)	21 (50)	3 (7)	11 (31)	21 (58)	4 (11)	20 (44)	20 (44)	5 (12)
alive with the disease	21 (22)	8 (42)	7 (37)	4 (21)	10 (67)	3 (20)	2 (13)	7 (33)	11 (52)	3 (14)
died from the disease	26 (27)	6 (27)	14 (64)	2 (9)	8 (33)	14 (58)	2 (8)	15 (60)	10 (40)	0 (0)
died from other causes	4 (4)	2 (50)	1 (25)	1 (25)	2 (50)	0 (0)	2 (50)	3 (75)	0 (0)	1 (25)

Variable	N (%)	*ERCC1* 19007T>C			*ERCC2* 2251A>C			*GSTPI* 313A>G		
		TT	CT	CC	AA	AC	CC	AA	AG	GG
T stage										
T1	1 (1)	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	1 [100)	0 (0)
T2	4 (4)	1 (33)	2 (67)	0 (0)	2 (50)	1 (25)	1 (25)	2 (50)	2 [50)	0 (0)
T3	64 (65)	23 (28)	30 (50)	7 (12)	22 (44)	24 (48)	4 (8)	29 (47)	26 [42)	7 (11)
T4	29 (30)	10 (42)	11 (46)	3 (12)	8 (32)	12 (48)	5 (20)	15 (52)	12 [41)	2 (7)
Sex										
male	59 (60)	21 (40)	27 (51)	5 (9)	**25 (49)**	**23 (45)**	**3 (6)**	**35 (59)**	**19 (33)**	**5 (8)***
female	39 (40)	13 (37)	17 (49)	5 (14)	**7 (24)**	**15 (52)**	**7 (24)****	**11 (30)**	**22 (59)**	**4 (11)**
Tumor location										
right	52 (54)	14 (30)	28 (61)	4 (9)	13 (33)	20 (51)	6 (15)	28 (56)	26 (32)	6 (12)
left	45 (46)	20 (49)	15 (37)	6 (15)	19 (47)	17 (43)	4 (10)	18 (40)	24 (53)	3 (7)
Median age (y)	64 (30–85)	65 (37-77)	64 (30-80)	56 (35-67)	63 (30–80)	64 (35–77)	64 (6–74)	62.5 (30–85)	64 (35–77)	66 (57–75)
Median follow-up period (mo)	37 (2–57)	38 (3-57)	36 (2-55)	37 (3-57)	38 (3–57)	30 (8–57)	33 (2–51)	36 (3–57)	37 (3–57)	42 (2 – 51)
Median time to progression (mo)	30 (2–57)	29 (3-57)	30 (2-55)	30 (3-49)	30 (3–57)	25 (4–55)	24 (2–51)	30.5 (3 – 57)	28 (3–55)	35 (2 – 51)

a Bold text indicates statistically significant differences.

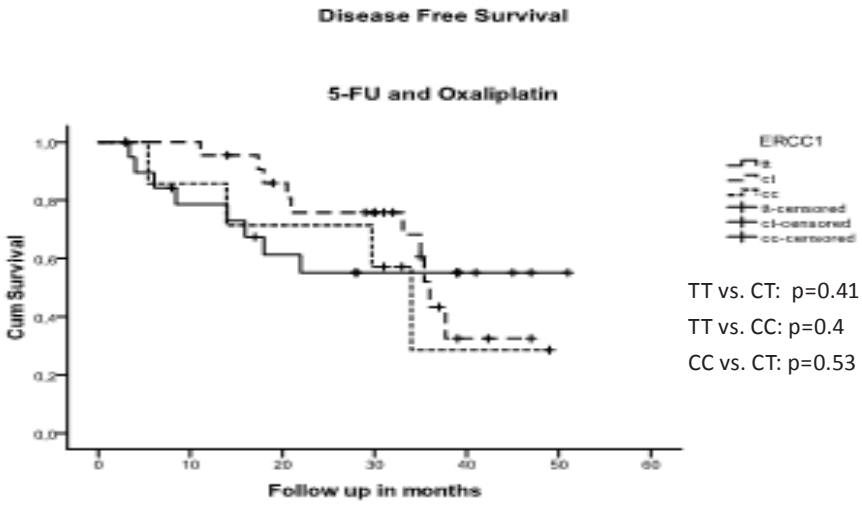
ERCC1 = excision repair cross-complementing rodent repair deficiency, complementation group 1; ***ERCC2*** = excision repair cross-complementing rodent repair deficiency, complementation group 2; ***GSTP1*** = glutathione S-transferase pi 1; **T** = tumor; * p = 0.02; ** p = 0.019.

Table 2: Cox proportional hazard model for disease-free survival in patients with the three SNPs that were tested.

Patient category and therapy	HR	95% CI	p-value
***ERCC1* 19007T>C**			
Fluorouracil + oxaliplatin (N = 48)			
male sex	2.65	0.94, 7.45	0.06
Age	0.97	0.91, 1.03	0.32
ERCC1 TT	Reference		
ERCC1 CT	0.67	0.23, 1.89	0.45
ERCC1 CC	0.94	0.26, 3.36	0.92
Fluorouracil + leucovorin (N = 36)			
male sex	0.69	0.25, 1.94	0.49
Age	0.97	0.91, 1.03	0.34
ERCC1 TT	Reference		
ERCC1 CT	1.42	0.51, 3.97	0.5
ERCC1 CC	1.95	0.31, 12.11	0.47
***ERCC2* 2251A>C**			
Fluorouracil + oxaliplatin (N = 43)			
male sex	2.16	0.64, 7.35	0.22
age	0.99	0.93, 1.07	0.96
ERCC2 AA	Reference		
ERCC2 AC	0.65	0.24, 1.8	0.41
ERCC2 CC	0.73	0.13, 4.11	0.72
Fluorouracil + leucovorin (N = 32)			
male sex	0.86	0.32, 2.34	0.77
Age	0.95	0.9, 0.99	0.04
ERCC2 AA	Reference		
ERCC2 AC	1.25	0.47, 3.33	0.65
ERCC2 CC	2.04	0.43, 9.68	0.37
***GSTPI* 313A>G**			
Fluorouracil + oxaliplatin (N = 50)			
male sex	2.8	0.97, 8.03	0.06
Age	0.96	0.91, 1.02	0.17
GSTPI AA	Reference		
GSTPI AG	2.1	0.84, 5.25	0.11
GSTPI GG	1.57	0.31, 7.9	0.59
Fluorouracil + leucovorin (N = 42)			
male sex	1.23	0.51, 2.97	0.65
Age	0.97	0.92, 1.02	0.23
GSTPI AA	Reference		
GSTPI AG	0.9	0.35, 2.28	0.80
GSTPI GG	0.41	0.05, 3.15	0.4

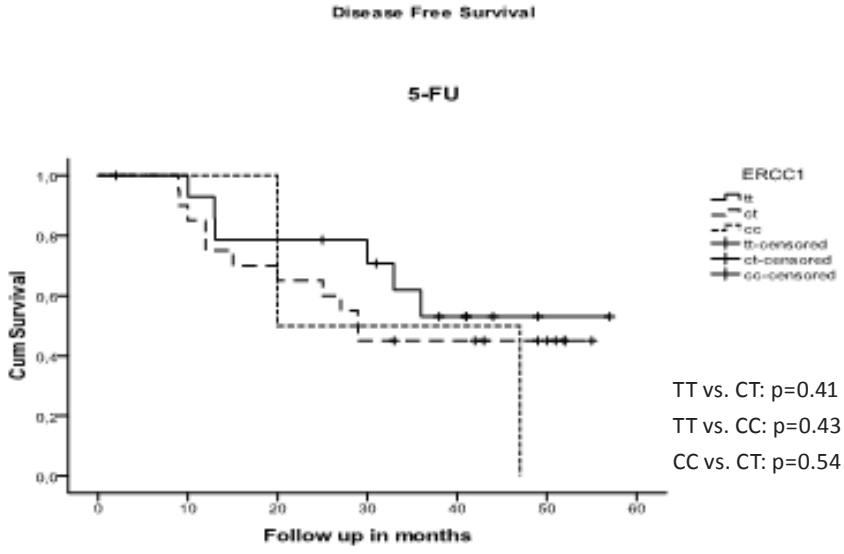
CI = confidence interval; ***ERCC1*** = excision repair cross-complementing rodent repair deficiency, complementation group 1; ***ERCC2*** = excision repair cross-complementing rodent repair deficiency, complementation group 2; ***GSTPI*** = glutathione S-transferase pi 1; **HR** = hazard ratio; **SNP** = single nucleotide polymorphism.

a)



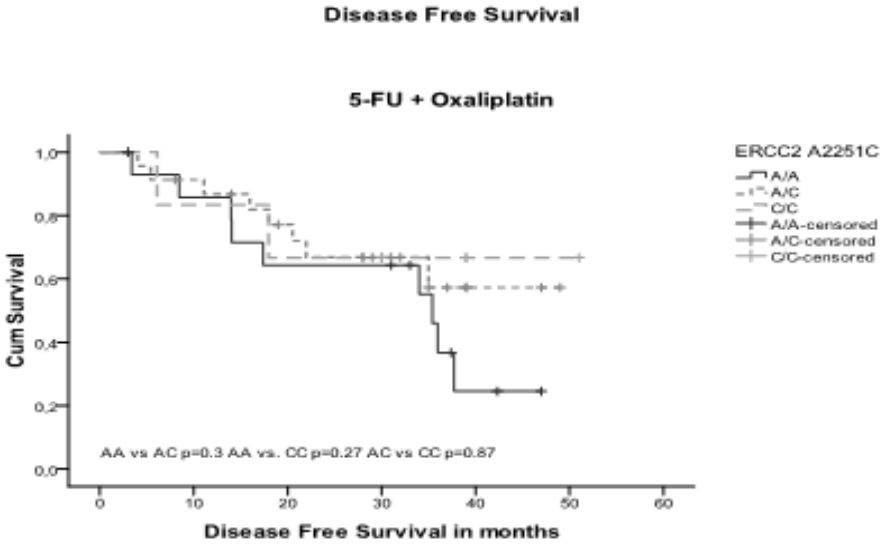
TT vs. CT: p=0.41
TT vs. CC: p=0.4
CC vs. CT: p=0.53

b)

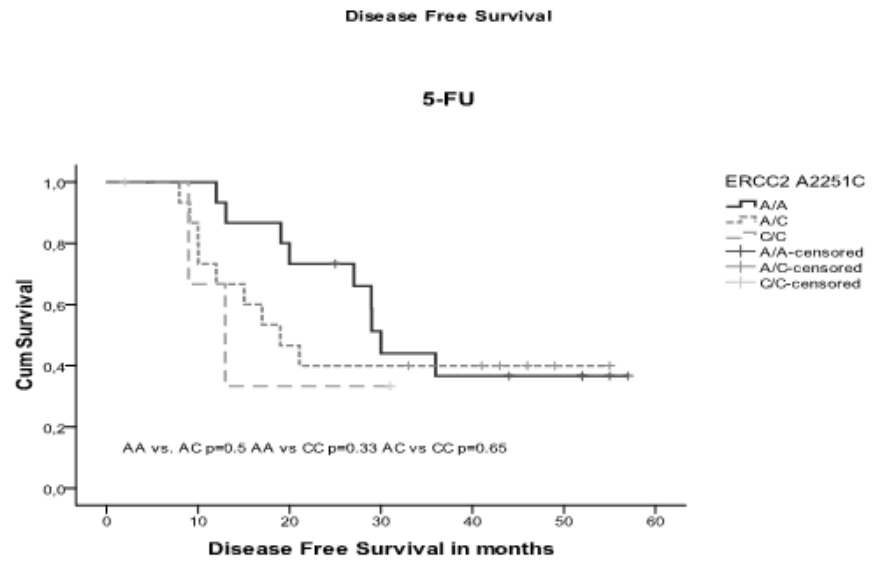


TT vs. CT: p=0.41
TT vs. CC: p=0.43
CC vs. CT: p=0.54

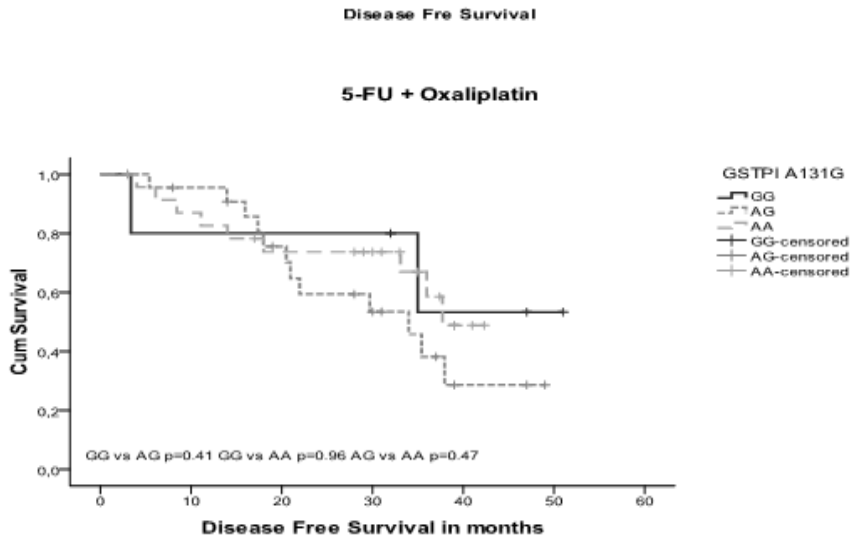
c)


Disease Free Survival
5-FU + Oxaliplatin
ERCC2 A2251C
A/A
A/C
C/C
A/A-censored
A/C-censored
C/C-censored
Cum Survival
1,0
0,8
0,6
0,4
0,2
0,0
AA vs AC p=0.3 AA vs. CC p=0.27 AC vs CC p=0.87
0
10
20
30
40
50
60
Disease Free Survival in months

d)


Disease Free Survival
5-FU
ERCC2 A2251C
A/A
A/C
C/C
A/A-censored
A/C-censored
C/C-censored
Cum Survival
1,0
0,8
0,6
0,4
0,2
0,0
AA vs. AC p=0.5 AA vs CC p=0.33 AC vs CC p=0.65
0
10
20
30
40
50
60
Disease Free Survival in months

e)



f)

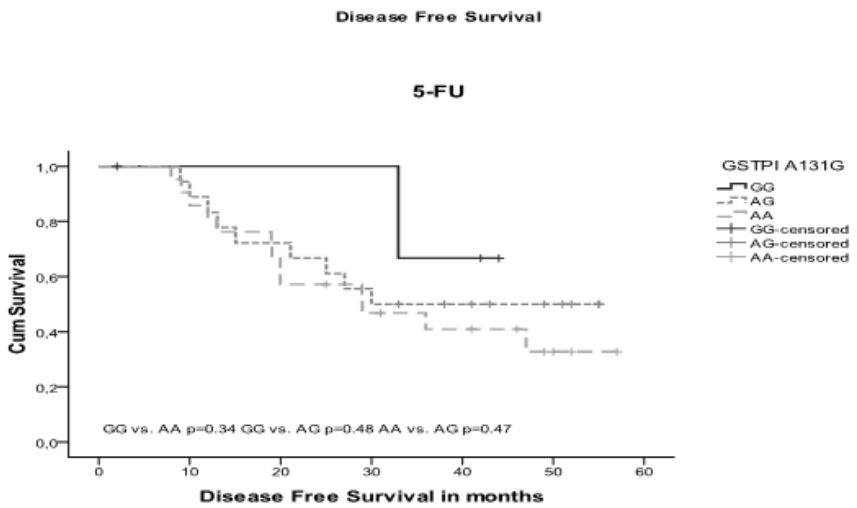


Figure 1: Kaplan-Meier plots for disease-free survival (**a**, **c** and **e**) in patients receiving combination therapy with fluorouracil and oxaliplatin, and (**b**, **d** and **f**) in patients treated with fluorouracil alone: (**a** and **b**) patients with the *ERCC1* 19007T>C SNP; (**c** and **d**) patients with the *ERCC2* 2251A>C SNP; and (**e** and **f**) patients with the *GSTP1* 313A>G SNP.

Discussion

The studied SNPs were selected on the basis of previously published results showing an association between these SNPs and oxaliplatin response in advanced colorectal carcinoma [8,9]. However, not all reports agree on the predictive value of these SNPs in oxaliplatin response. Moreover, most research has been performed in advanced colorectal carcinoma where therapy is palliative; therefore, in this study, we aimed to determine the effects of the *ERCC1* 19007T>C*, ERCC2* 2251A>C, and *GSTPI* 313A>G polymorphisms in response to oxaliplatin administered in an adjuvant setting. In order to answer this question, survival according to the different SNPs and the therapy given were analyzed in a cohort of patients with stage III disease. From the present data, it can be concluded that none of the SNPs studied here are good markers of response to oxaliplatin given in the adjuvant setting.

In a recent meta-analysis, Yin *et al.* concluded that *ERCC1* 19007T>C had predictive value as a marker of response to oxaliplatin therapy, however, their analysis included studies of gastric cancer as well as colorectal cancer [9]. Considering colorectal cancer solely, the existing findings on the predictive value of *ERCC1* 19007T>C are rather inconclusive. Taking previous findings together with the data presented in this study, it can be concluded that *ERCC1* 19007T>C is not a reliable marker of response to oxaliplatin in colon carcinoma patients [8,18,19].

Our findings on the *ERCC2* 2251A>C SNP showed that this SNP is not a reliable predictive marker of response to adjuvant oxaliplatin therapy either. The predictive value of this polymorphism is controversial; some researchers have found that the A allele is predictive of a better outcome [8,14,15], whereas others have demonstrated the opposite [10,11]. In the present cohort of patients, no effects of *ERCC2* 2251A>C were seen. A possible explanation for these contradictory findings could be that all of these studies, including ours, were retrospective studies, and most of them were conducted in metastatic colorectal cancer patients treated with several lines of therapy. Thus, prospective studies in homogeneous patient populations must be carried out to elucidate the clinical value of these markers.

Finally, although the findings on the *GSTPI* gene polymorphism in this study contradict those in other reports [8], they corroborate the findings of Kweekel *et al*. [22] in stage IV colon cancer. Therefore, we conclude that it is likely that the *GSTP1* 313A>G SNP has no predictive value in colorectal cancer therapy.

In our study, certain genotypes were associated with sex and conferred worse overall survival in males than in females. Sex is a well known prognostic factor in colorectal cancer, and the reasons for sex-related survival advantages are not yet fully understood[23-25]. The association found here between certain polymorphic alleles and sex – and the possible effects of the polymorphisms on cancer susceptibility, prognosis, and response to therapy – could at least partly explain sex-related survival differences. Recently, it has been proposed that sex should be taken into account when evaluating predictive markers of response, as these markers have been found to be different for males and females [26].

Finally, we would like to stress the importance of prospective studies with enough statistical power to confirm whether or not these SNPs have value as predictive markers in clinical practice.

None of the polymorphisms studied in the present project seemed to be a reliable predictive marker of response to adjuvant oxaliplatin therapy. It seemed, however, that the *GSTPI* 313A>G homozygous A genotype had a prognostic effect in male patients, independently of the treatment given.

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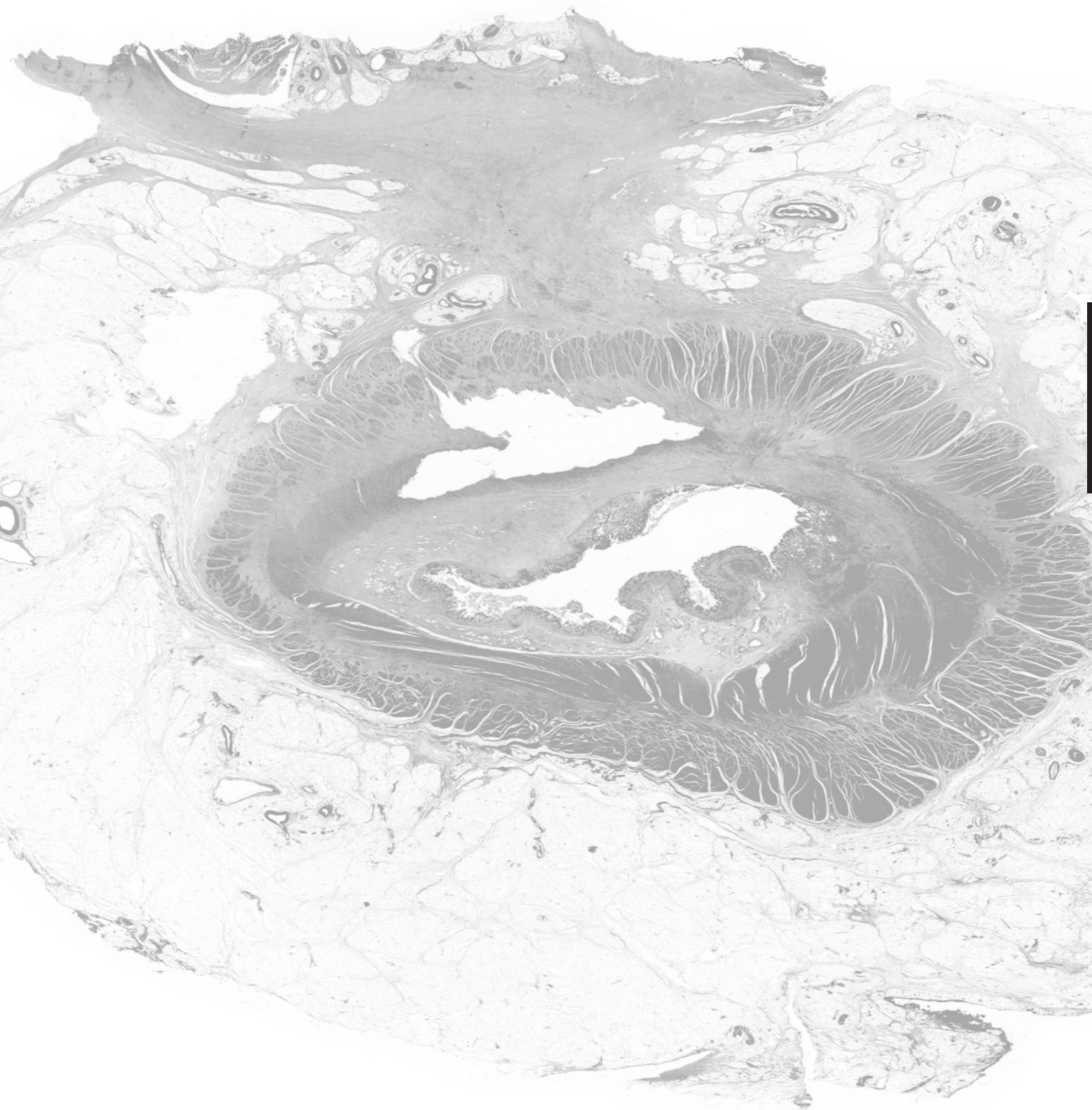

5

The *BRAF* V600E mutation is an independent prognostic factor for survival in stage II and stage III colon cancer patients

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Abstract

Molecular markers in colon cancer are needed for a more accurate classification and personalized treatment. We determined the effects on clinical outcome of the *BRAF* mutation, microsatellite instability (MSI) and *KRAS* mutations in stage II and III colon carcinoma.

Stage II colon carcinoma patients (n=106) treated with surgery only and 258 stage III patients all adjuvantly treated with 5-FU chemotherapy, were included. *KRAS* mutations in codons 12 and 13, V600E *BRAF* mutation and MSI status were determined.

Older patients ($p<0.001$), right sided ($p=0.018$), better differentiated ($p=0.003$) and MSI tumors ($p<0.001$) were significantly more frequent in stage II than stage III.

In both groups, there was a positive association between mutated *BRAF* and MSI ($p=0.001$) and *BRAF* mutation and right sided tumors ($p=0.001$). Mutations in *BRAF* and *KRAS* were mutually exclusive.

In a multivariate survival analysis with pooled stage II and III data *BRAF* mutation was an independent prognostic factor for overall survival and cancer specific survival (HR=0.45 95%CI 0.25 – 0.8 for OS and HR=0.47 95%CI 0.22 – 0.99). *KRAS* mutation conferred a poorer DFS (HR=0.6 95%CI 0.38 – 0.97).

The V600E *BRAF* mutation confers a worse prognosis to stage II and III colon cancer patients independently of disease stage and therapy.

Introduction

Colon carcinoma is classified according to clinical and histopathological criteria. Prognosis and therapy relate to this classification. According to the Dutch treatment guidelines previous to 2006, stage II patients were solely treated with surgery. Stage III patients would receive adjuvant chemotherapy after surgery. Around 20% of stage II patients will develop a relapse in the first five years after surgery. Probably, this group of patients would benefit from adjuvant chemotherapy. On the other hand, 60% of stage III patients are cured after surgery and do not benefit from the adjuvant treatment [1,2]. Hence, other criteria for adjuvant therapy are needed. Molecular markers might prove to be better than clinical and histopathological criteria for therapy selection.

Microsatellite instability (MSI) and *KRAS* mutations have been widely studied in colorectal cancer. Around 20% of the sporadic colon cancers show MSI due to defects in the mismatch repair system (MMR). MSI is associated with a better prognosis[3-6]. Approximately 35% of colon cancers carry a mutation in codons 12 or 13 of the *KRAS* gene leading to the constitutive activation of its downstream pathway and to uncontrolled cell division [7-9]. *BRAF* is recently being studied in relation to prognosis[10-13]. *BRAF* is a downstream effector molecule of *KRAS*. 90% of the *BRAF* mutations consist in a valine to glutamate transition at position 600 of the protein, the so called V600E mutation, which causes the constitutive activation of the protein. This mutation is found in approximately 20% of the colonic tumors.

Mutations in *BRAF* and in *KRAS* are mutually exclusive. Tumors harboring the V600E *BRAF* mutation have other clinical and histopathological features than *KRAS* mutated tumors [14].

The value of *KRAS* mutations in stage II and III is unknown. *BRAF* has been studied only in heterogeneous colon carcinoma patients cohorts including all disease stages [10-12] and recently in a group of stage IV colorectal cancer [13]. To date, it remains unknown what the effect of the *BRAF* mutation is on clinical outcome of patients with either stage II or III disease.

In this study we aimed to determine the status of the V600E *BRAF* mutation and other molecular markers, like MSI status and *KRAS* mutations in two well defined groups of stage II and III colon carcinoma patients who were treated according to the Dutch guidelines previous to 2006 and to assess their effect on patient outcome.

Patients and Methods

Patient population

Three hundred sixty four patients diagnosed at the PAMM Laboratory for Pathology in Eindhoven, the Netherlands and treated in four different regional hospitals in the south of the Netherlands, between 1996 and 2004, were included in this study. We included 106 patients diagnosed with stage II colon carcinoma and treated with surgery only and 258 stage III disease patients treated with surgery followed by adjuvant 5-FU in combination with leucovorin chemotherapy like established by the Dutch guidelines for the treatment of colon cancer previous to 2006. A tumor was considered right sided when it was located between the coecum and the splenic flexure. The remaining tumors were considered left sided. Rectal tumors were not included. Demographic and clinical data on the patients were facilitated by the Cancer Registry of the Comprehensive Cancer Centre South (IKZ, Eindhoven, the Netherlands). In over 93% of the patients data was complete. Follow-up was obtained from the available medical records of the patients.

The use of clinical material for this retrospective study was approved by the institutional review board according to the guidelines of the Dutch Federation of Research Associations.

From all patients with sufficient available material, tumor DNA was isolated. For this purpose, a tumor area with at least 30% tumor cells from glass slide according to HE stained sections was selected by an experienced pathologist. Subsequently, the selected areas were macrodissected from archival paraffin embedded tissue. DNA was purified after proteinase K digestion with the HPPTP kit (Roche, Almere, the Netherlands) following manufacturer's instructions.

From 76 patients data were missing due to different reasons, firstly some tissue blocks were not present in our archive (47.4%), secondly some samples did not reach 30% tumor cells (43.4%) and additionally not all DNA samples could be amplified by PCR (9.2%).

Molecular characterization

BRAF mutation analysis

The V600E mutation on the *BRAF* gene was detected by means of real time PCR using

the following primers and probes, forward 5'CTA CTG TTT TCC TTT ACT TAC TAC ACC TCA GA 3' and reverse 5'ATC CAG ACA ACT GTT CAA ACT GAT G 3', wild type probe VIC-5'CTA GCT ACA GTG AAA TC 3' and mutant probe FAM-5'TAG CTA CAG AGA AAT C 3' like described elsewhere[15]. A PCR product of 136 bp was obtained. The assay showed to have a detection limit of at least 10% tumor cells in a given specimen. All PCR reactions were performed on the Light Cycler v2.0 (Roche, Almere, the Netherlands) using Roche chemistry in a total volume of 20 microliters.

Microsatellite instability

Microsatellite instability was detected using only one marker of the Bethesda panel, i.e. the mononucleotide repeat BAT26. This marker was chosen because in the Caucasian race, it detects 99% of the MSI high patients and normal DNA is not necessary [16,17]. PCR was performed using the following primers, forward VIC-5´TGA CTA CTT TTG ACT TCA GCC 3´ and reverse 5´ACC CAT TCA ACA TTT TTA ACC C 3´. The expected product length is 116 bp. Subsequently, PCR products were diluted depending on their intensity and denatured using formamide and incubated at 95°C for 3 minutes. Products size were analyzed using the ABI3130 (Applied Biosystems, Nieuwerkerk aan de Ijssel, the Netherlands) and GeneMapper 4.0 software package.

KRAS mutation analysis

Mutations in codons 12 and 13 of the *KRAS* gene were detected by DNA sequencing. Briefly, PCR amplification of the cited codons was performed using the following primers; forward 5´AGG CCT GCT GAA AAT GAC TG 3´and reverse 5´TCA AAG AAT GGT CCT GCA CC 3´ as previously described by van Zandwijk et al [18]. The expected product length was 172 bp. After purification of the PCR product, the sequence reaction was performed using the same primers independently and the Big Dye reagents (Applied Biosystems, Nieuwerkerk aan de Ijssel, the Netherlands). Products were separated on the ABI3130 (Applied Biosystems, Nieuwerkerk aan de Ijssel, the Netherlands). The sequences were evaluated with the Sequencing Analysis 5.3.1 software.

Statistical Analysis

SPSSv.16 software for Windows (Chicago, IL) was used. X2, Fischer exact tests and Student's t-test were used to analyze the relationship between variables.

Stage II and stage III groups were first analyzed separately and pooled during survival

analysis to increase the sensitivity of the tests. Univariate survival analysis was performed with Kaplan Meier analysis and survival curves were compared by Log-Rank tests. Multivariate analysis was performed with Cox Proportional Hazards regression analysis. T and N stage, but also age, sex, tumour location, differentiation grade, *BRAF*, *KRAS*, and MSI status were included in the model. In case of statistical significant interaction between these variables in the model, we would stratify the analyses accordingly. We considered a minimum of 10 to 15 events per predictor necessary to proceed with multivariate survival analyses [19]. In order to avoid overfitting, all variables were entered and maintained in the model, e.g. not using automated stepwise regression. For the same reason, those variables which did not exhibit a statistically significant relation with survival in the univariate analysis were also entered into the model. Besides, variables in isolation may behave quite differently with respect to the response variable when they are considered simultaneously with 1 or more other variables [20]. Overall survival (OS) was defined as the time between diagnosis and either death of disease or death of other cause, whenever this was specified in the patients' medical record. Disease free survival (DFS) was defined as the time between diagnosis and disease recurrence or development of distant metastasis. Finally, cancer specific survival (CSS) was defined as the period of time between diagnosis and death due to the disease.

Results

Patients' demographic and clinicopathological characteristics

Patients' characteristics according to stage are shown in table 1.

By definition none of the patients diagnosed with stage II disease had tumor positive lymph nodes whereas all of the stage III patients had positive lymph nodes. In both groups a similar number of lymph nodes were examined for diagnosis, median number of 7 in stage II and of 8 in stage III.

In the stage II group median age was 73 years (range 30-94) whereas in the stage III group it was 64 years (range 30-84). This difference was statistically significant ($p<0.001$).

The tumor location was also significantly different between groups, 68% right sided tumors in stage II vs. 54% in stage III ($p=0.018$). Well or moderately differentiated tumors were more frequent in stage II patients than in stage III (87% in stage II vs. 72% in stage III, $p=0.005$).

The cause of death was significantly different between groups. In the stage II group 30% of the patients had died because of reasons other than cancer (as specified in their medical records) and 10% due to cancer related reasons. In the stage III group only 7% had died of non-cancer related causes and 32% died due to cancer related causes ($p<0.001$).

Median follow up of the stage II group was 55 months (0-109) and 46 months (2-133) for the stage III group.

KRAS, *BRAF* and MSI status

Table 2 a&b shows the frequencies of the different mutations in the patient population and the significant associations between variables for the two patients' populations. The percentages of the mutations in *KRAS* and *BRAF* did not differ between the two populations. *KRAS* mutations were found in 33% of stage II patients vs. 35% of stage III. *BRAF* was mutated in 22% of stage II and in 19% of stage III patients. However, the proportion MSI tumors was significantly higher in the stage II group than in stage III (25% vs. 14%, respectively, $p=0.024$).

KRAS and *BRAF* mutations were mutually exclusive ($p<0.001$) in both populations. There was no significant association between *KRAS* mutations and the development of a distant metastasis or local relapse in stage II patients ($p=0.08$). Moreover, it did reach

statistical significance in stage III patients (p=0.014). *KRAS* mutations were associated to better differentiated tumors (p=0.013 stage II and p=0.06 stage III).
The carriage of the V600E *BRAF* mutation was significantly associated with MSI (p<0.001), right side location (p<0.001) in both populations.
In both groups MSI tumors were right sided (p=0.003 stage II and p<0.001 stage III) and poorly differentiated (p=0.024 stage II and p=0.022 stage III).

Survival analysis

In a univariate analysis, in both groups separately the *BRAF* V600E mutation was significantly associated with a shorter CSS in stage II disease (p=0.022) but not in stage III disease (Figure 1). In both groups there was a trend towards a longer OS for the carriers of wild type *BRAF* (p=0.194 stage II and 0.069 stage III) (Figure 2). DFS was not significantly different between *BRAF* mutants and wild type tumors.
When stratifying for MSI status, *BRAF* mutation resulted in shorter survival in MSS patients in both stage II and stage III disease (p=0.011 stage II CSS and p=0.016 stage III OS), but not in the MSI group.
In the stage III group, *KRAS* mutations seemed to confer a significantly worse DFS than *KRAS* wild type (p=0.03) (Figure 3). This effect was not present in the stage II group.

Multivariate analysis

Since results did not significantly differ between both populations, data of both groups were pooled in order to increase sensitivity of the multivariate analysis. A Cox Proportional Hazards model including differentiation grade, age as a continuous variable, sex, tumor location, T-stage, N-stage, *KRAS* status, *BRAF* status and MSI status was used. The results of this model are shown in table 3. Therapy was not included in the model because it covariates linearly with N-stage.
BRAF mutation was as an independent factor for a shorter OS (HR=0.45 95%CI 0.25-0.8), DFS (HR=0.43 95%CI 0.22-0.82) and CSS (HR=0.47 95%CI 0.22-0.99). *KRAS* mutation was an independent prognostic factor for a shorter DFS (HR=0.6 95%CI 0.4-0.97). T-stage was a prognostic factor for DFS, OS and CSS. N-stage, as positive or negative lymphnodes, was prognostic for DFS and CSS. Finally, male gender was a significant variable for a shorter OS (HR=1.84 95%CI 1.19-2.85).

Table 1: Clinicopathological characteristics in stage II and III patients.

Characteristics	Stage II N(%)	Stage III N(%)	p-value
Sex			
Male	54 (51)	144 (56)	0.42
Female	52 (49)	114 (44)	
Location			
Right	69 (68)	137 (54)	**0.018**
Left	33 (32)	117 (46)	
Age			
Mean	71.5	62.5	**<0.001**
Median	73	64	
T-stage			
T1	0	2 (0.8)	0.06
T2	3 (3)	22 (8.5)	
T3	85 (82.5)	186 (72)	
T4	15 (14.5)	48 (18.7)	
Differentiation grade			
Well/moderate	85 (87)	177 (72.5)	**0.005**
Poor/Undifferentiated	13 (13)	67 (27.5)	
Follow up status			
No evidence of disease	52 (50.5)	124 (48.6)	**<0.001**
Alive with disease	10 (9.7)	31 (12.2)	
Death of disease	10 (9.7)	83 (32.5)	
Death of other cause	30 (29.1)	17 (6.7)	

Table 2 a: Patient's characteristics according to disease stage. (wt=wild type mut=mutated).

a) stage II

Stage II		*BRAF*			*KRAS*			MSI		
	N (%)	wt	mut	p	wt	mut	p	MSI	MSS	p
Sex										
Male	54 (51)	38 (81)	9 (19)	0.6	33 (70)	14 (30)	0.5	11 (23)	37 (77)	0.8
Female	52 (49)	35 (76)	11 (24)		28 (62)	17 (38)		12 (26)	34 (74)	
Location										
Right	69 (68)	46 (72)	18 (28)	**0.01**	42 (67)	21 (33)	1.0	21 (32)	44 (68)	**0.003**
Left	33 (32)	25 (96)	1 (4)		18 (69)	8 (31)		1 (4)	25 (96)	
Age										
0-59	12 (12)	11 (100)	0 (0)	0.17	8 (73)	3 (27)	0.4	0 (0)	11 (100)	0.25
60-66	17 (16.5)	12 (80)	3 (20)		9 (60)	6 (40)			11 (73)	
67-72	19 (18.5)	11 (65)	6 (35)		14 (83)	3 (18)		4 (27)	12 (71)	
≥73	55 (53)	39 (78)	11 (22)		30 (61)	19 (39)		5 (29)	37 (72)	
								14 (28)		
Median age	73									
T-status							0.4			
T1	0 (0)				0	0				
T2	3 (3)	2 (67)	1 (33)	0.36	3 (100)	0 (0)		2 (67)	1 (33)	0.24
T3	85 (82.5)	62 (80.5)	15 (19.5)		49 (64.5)	27 (35.5)		18 (23)	59 (77)	
T4	15 (14.5)	9 (64)	5 (36)		10 (71)	4 (29)		4 (27)	11 (73)	
N-status										
N-	106 (100)	73 (78)	21 (22)		62 (67)	31 (33)		24 (25)	71 (75)	
N+	0				0	0		0	0	
Differentiation							**0.013**			0.024
Well/Moderate	25 (25)	63 (81)	15 (19)	0.21	46 (60.5)	30 (39.5)		16 (20)	62 (80)	
Poor/Undiff.	13 (13)	6 (60)	4 (40)		10 (100)	0 (0)		6 (55)	5 (45)	
BRAF							**<0.001**			**<0.001**
wt	73 (78)				41 (57)	31 (43)		8 (11)	65 (89)	
mut	21 (22)				21 (100)	0 (0)		15 (71)	6 (29)	
KRAS				**<0.001**						**0.001**
wt	62 (67)	41 (66)	21 (34)					22 (26)	40 (64)	
mut	31 (33)	31 (100)	0 (0)					1 (3)	30 (97)	
MSI status				**<0.001**			**0.001**			
MSI	24 (25)	8 (35)	15 (65)		22 (96)	1 (4)				
MSS	71 (75)	65 (91.5)	6 (8.5)		40 (57)	30 (43)				

Table 2 b: Patient's characteristics according to disease stage. (wt=wild type mut=mutated).

b) stage III

Stage III		*BRAF*			*KRAS*			MSI		
	N (%)	wt	mut	p	wt	mut	p	MSI	MSS	p
Sex										
Male	144 (56)	95 (83)	20 (17)	0.6	75 (67)	37 (33)	0.65	16 (13)	103 (87)	0.7
Female	114 (44)	70 (79.5)	18 (20.5)		55 (63)	32 (37)		14 (16)	76 (84)	
Location										
Right	137 (54)	76 (70)	32 (30)	**<0.001**	67 (63)	39 (37)	0.55	27 (24)	84 (76)	**<0.001**
Left	117 (46)	89 (95)	5 (5)		62 (67)	30 (33)		2 (2)	94 (98)	
Age										
0-59	83 (32)	51 (82)	11 (18)	0.9	45 (74)	16 (26)	0.3	12 (18)	54 (82)	0.7
60-66	82 (32)	59 (83)	12 (17)		44 (65)	24 (35)		8 (11)	63 (89)	
67-72	61 (24)	36 (78)	10 (22)		28 (61)	18 (39)		7 (15)	40 (85)	
≥73	32 (12)	19 (79)	5 (21)		13 (54)	11 (46)		3 (12)	22 (88)	
Median age	64									
T-status										
T1	2 (0.8)	1 (100)	0 (0)	0.9	0 (0)	1 (100)	0.105	0 (0)	1 (100)	0.6
T2	22 (8.5)	14 (82)	3 (18)		14 (82)	3 (18)		1 (6)	17 (94)	
T3	186 (72)	120 (82)	27 (18)		88 (61.5)	55 (38.5)		22 (14)	131 (86)	
T4	48 (18.7)	30 (79)	8 (21)		28 (74)	10 (26)		7 (19)	30 (81)	
N-status										
N-	0	0	0		0	0		0	0	
N+	255	163 (81)	38 (19)		127 (65)	69 (35)		30 (14)	178 (86)	
Differentiation										
Well/Moderate	29 (12)	121 (87)	18 (13)	**<0.0001**	85 (62.5)	51 (37.5)	**0.036**	16(20.5)	62 (79.5)	**0.022**
Poor/Undiff.	66 (27)	35 (64)	20 (36)		41 (77)	12 (23)		15(10)	129 (90)	
BRAF										
wt	165 (81)				94 (58)	69 (42)	**<0.001**	14 (9)	149 (91)	**<0.001**
mut	38 (19)				36 (100)	0 (0)		14 (37)	24 (63)	
KRAS										
wt	130 (65)	94 (72)	36 (28)	**<0.001**				25 (19)	103 (81)	**0.001**
mut	69 (35)	69 (100)	0 (0)					2 (3)	67 (97)	
MSI status										
MSI	30 (14)	14 (50)	14 (50)	**<0.001**	25 (93)	2 (7)	**0.001**			
MSS	179 (86)	149 (86)	24 (14)		103 (61)	67 (39)				

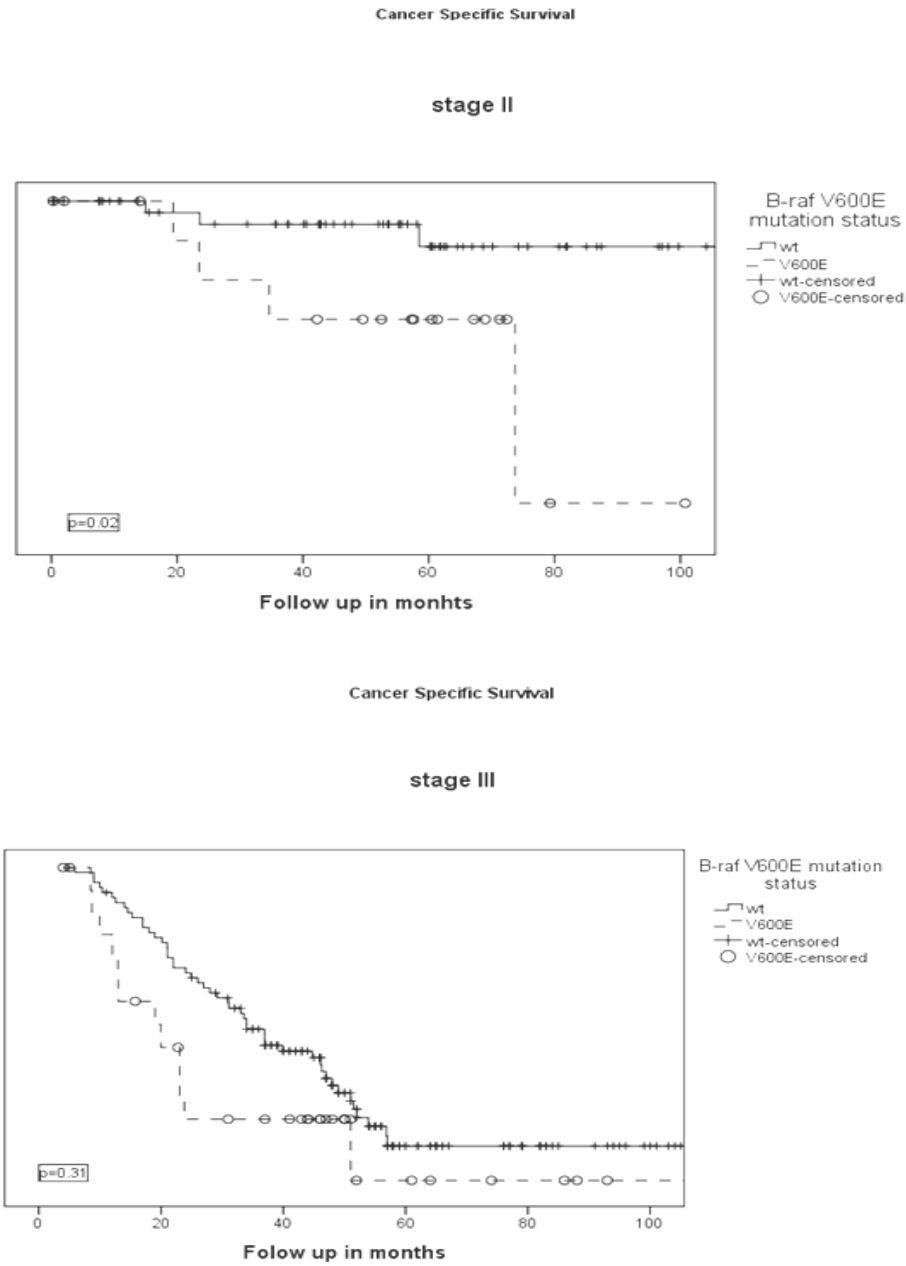

Figure 1: Kaplan Meier plots for CSS in stage II and in stage III patients according to *BRAF* V600E mutational status.

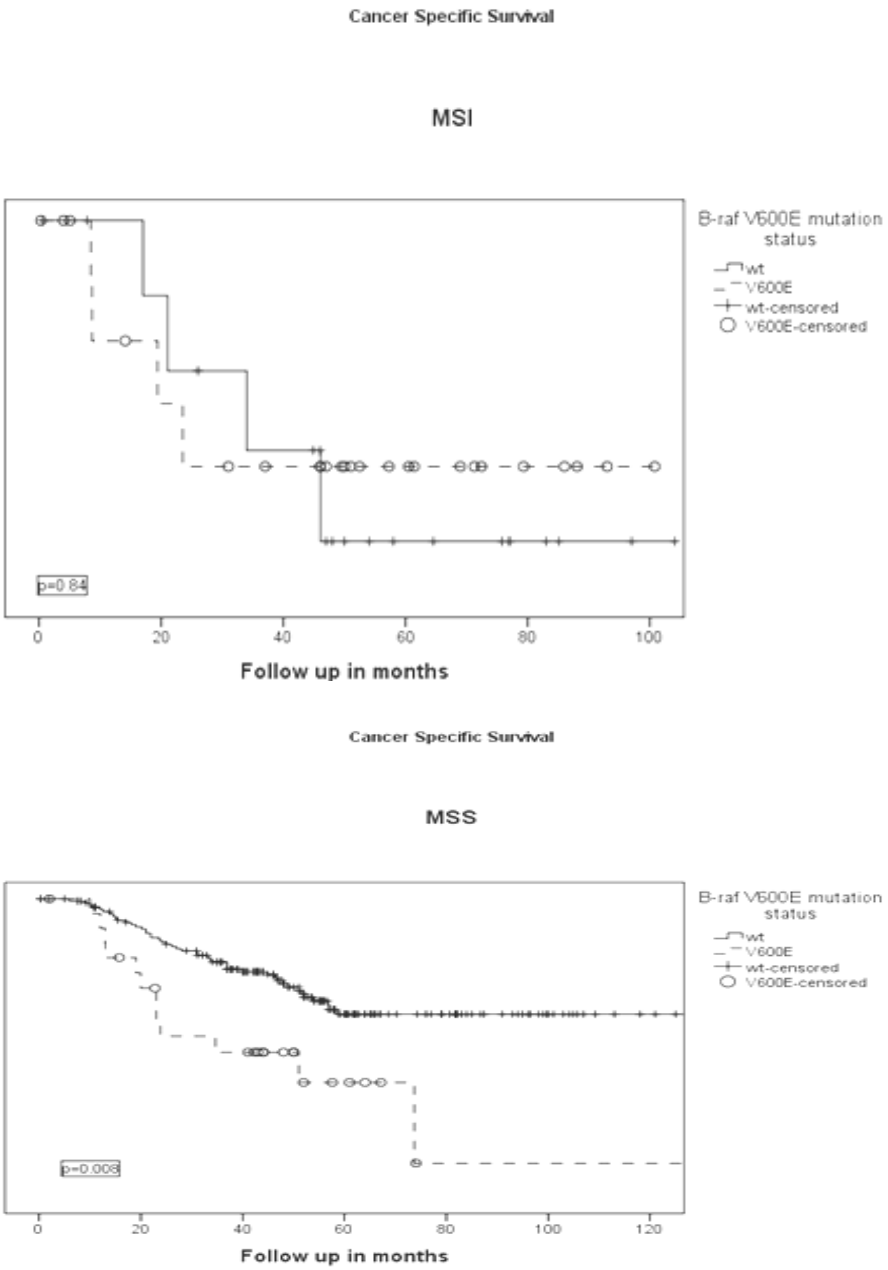

Figure 2: Kaplan Meier plots for CSS according to *BRAF* V600E mutational status in the whole group stratified according to MSI status of the tumor.

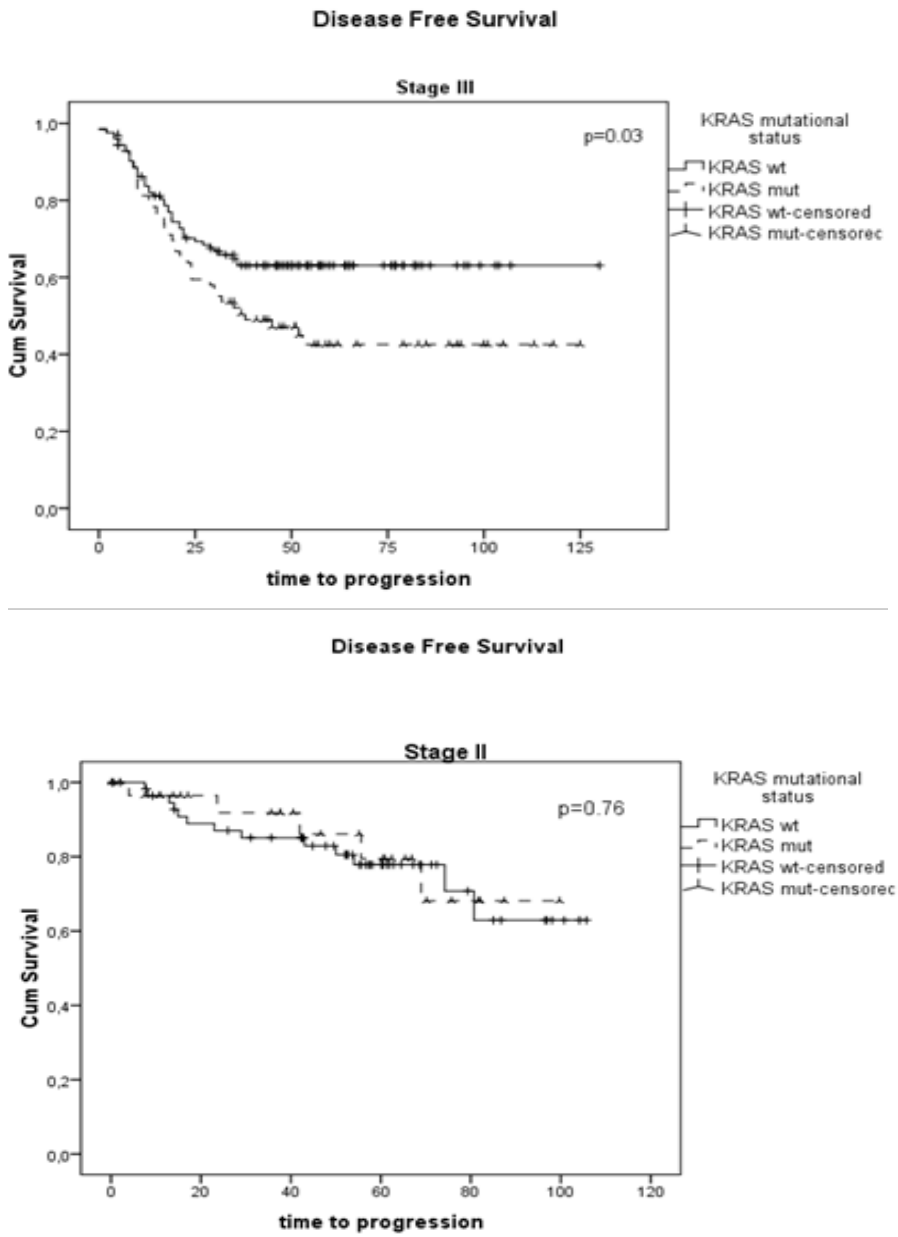

Figure 3: Kaplan Meier plots for DFS according to *KRAS* mutational status in stage II and III independently.

Table 3: Cox proportional hazards model for overall survival, disease free survival and cancer specific survival.

	Overall survival		Disease Free Survival		Cancer Specific Survival	
	HR	(95% CI)	HR	(95% CI)	HR	(95% CI)
Age	1.009	(0.98 – 1.03)	0.99	(0.97 – 1.02)	0.99	(0.97 – 1.02)
Sex	1.84	**(1.19 – 2.85)***	1.1	(0.72 – 1.7)	1.25	(0.73 – 2.15)
Location	0.71	(0.45 – 1.13)	0.7	(0.44 – 1.09)	0.74	(0.42 – 1.31)
***KRAS* status**						
wt	0.83	(0.51 – 1.35)	0.6	(0.38 – 0.97)	0.72	(0.39 – 1.3)
mut	1	(referent)	1	(referent)	1	(referent)
***BRAF* status**						
wt	0.45	**(0.25 – 0.8)***	0.43	(0.22 – 0.82)	0.47	**(0.22 – 0.99)***
mut	1	(referent)	1	(referent)	1	(referent)
MSI status	0.8	(0.42 – 1.5)	0.49	(0.23 – 1.05)	0.6	(0.22 – 1.4)
T stage						
T2	0.2	**(0.04 – 0.8)***	0.07	**(0.009 – 0.51)***	0.001	(0 - >1000)
T3	0.5	**(0.30 – 0.8)***	0.49	**(0.3 – 0.8)***	0.42	**(0.24 – 0.75)***
T4	1	(referent)	1	(referent)	1	(referent)
N-stage						
N-	0.93	(0.56 – 1.52)	0.5	**(0.3 – 0.9)***	0.3	**(0.12 – 0.72)***
N+	1	(referent)	1	(referent)	1	referent
Differentiation grade						
Well/Moderate	0.85	(0.51 – 1.40)	0.99	(0.59 – 1.6)	0.67	(0.37 – 1.22)
Poor/Undifferentiated	1	(referent)	1	(referent)	1	(referent)

* $p<0.05$

OS N=261, CSS & DFS N=252

Discussion

The molecular signature of a tumor will most likely influence patient survival. In stage II and III colon cancer the use of molecular markers might be particularly important in order to offer the most adequate therapy to each patient and avoid unnecessary chemotherapeutic treatment. In this study, we assessed the effect of the V600E *BRAF* mutation, *KRAS* mutations and MSI on patient outcome, in two well defined colon cancer populations of stage II and III patients.

In our population, the V600E *BRAF* mutation is an independent prognostic factor. The carriage of the mutation accounts for a significantly higher risk of dying of cancer related causes, independently of other factors like age, sex, location of the tumor, MSI status, *KRAS* mutational status, differentiation grade, T-stage and N-stage.

Our results agree with recent published studies from Ogino *et al.* and Tol *et al*. However, Ogino *et al.* found a relationship between *BRAF* mutation and CSS in an heterogeneous group of colon cancer patients including all disease stages [11], whereas, our study focus solely on a well described homogeneous stage II and III group. On the other hand, Tol *et al.* demonstrated a positive correlation between the V600E *BRAF* mutation and a shorter survival in a group of metastatic colorectal patients independently of the treatment arm (capecitabine, oxaliplatin, bevacizumab with or without cetuximab) [13]. However, the patients included in that study did all receive palliative chemotherapy and therefore no conclusion could be drawn about either the prognostic or predictive value of the *BRAF* mutation. From our data, we can conclude that the *BRAF* mutation is an independent prognostic factor in all patients with stage II and III colon carcinoma. It could be argued that our selection of patients based on the therapy according to the guidelines could bias the results. However, identical results were obtained in a larger group including stage III patients who did not receive adjuvant chemotherapy (data not shown).

Moreover, concordant with the literature [10,12], the V600E *BRAF* mutation identifies a small group of patients with microsatellite stable tumors who had a poor survival. However, the interaction between MSI, *BRAF* and disease outcome remains subject of study since in the multivariate analysis, MSI seemed to play a marginal role depending on therapy in patients' survival.

The presence of a *KRAS* mutation did not have any effect on patient overall survival in stage II and III disease. However, there was significant difference in DFS between

KRAS mutated and wild type tumors. The prognostic value of *KRAS* mutations in stage II and III colon carcinoma remains controversial. Many studies have reported a prognostic role for *KRAS* and many others failed to report this effect, as reviewed by Castagnola[21]. Based on our results we can conclude that *KRAS* seems to play a role in disease progression, mainly in stage III colon cancer patients, this effect is absent in stage II patients.

In our study, a group of stage II patients, who did not receive adjuvant therapy after surgery and a group of stage III patients who did receive 5-FU based adjuvant chemotherapy according to the Dutch guidelines previous to 2006 were selected. This treatment selection is the major reason for the differences in age and follow up status between patients in the two groups. It is known that only younger patients with a good general condition and little co-morbidity are offered adjuvant chemotherapy. Since all stage III patients in our group received chemotherapy, they were younger and had less comorbidity and thus less non-cancer related deaths than stage II patients, who frequently died of non cancer related deaths like heart failure.

Other significant differences between the two groups were the frequency of MSI and of right sided tumors in the stage II group. For the MSI determination, we choose the mononucleotide repeat BAT [26], because it discriminates 99% of MSI in the Caucasian population without the requirement of amplified normal DNA, like previously described[17]. The use of only one marker could have diminished the sensitivity of our analysis but not the specificity [16,17]. The higher frequency of MSI tumors in stage II is probably due to the significant association of MSI and right sided tumors and the higher proportion of these tumors among stage II patients which in turn can be explained by the shift in tumor location that occurs as patient age increases [22].

Due to the retrospective character of this study, we were not able to test patients who were treated according to the recently published Dutch guidelines where a difference in treatment is made between stage II and high risk stage II. Since 2006, high risk stage II patients receive adjuvant chemotherapy after surgery. High risk stage II patients are defined as having pT4 lesions, lymphovascular invasion, tumor perforation or obstruction, poorly differentiated histology, or less than 10 lymph nodes removed. Eighty four percent of our stage II patients would be nowadays considered as high risk patients. The majority due to the insufficient number of lymph nodes examined. Therefore, we can conclude that the negative effects of the V600E *BRAF* mutation on survival are applicable to this group of patients and that this mutation can be

considered as a prognostic marker.

In conclusion, *BRAF* is an independent prognostic factor in stage II and III colon cancer. These results are promising for the treatment of colon cancer patients since determination of the V600E *BRAF* mutation can discriminate between patients who have a shorter OS, DFS and CSS. The exact effect of MSI and of *KRAS* on survival should be further elucidated. In contrast, this *BRAF* mutation might become an important molecular marker in the future for drug development and in the decision making for patient tailored adjuvant therapy.

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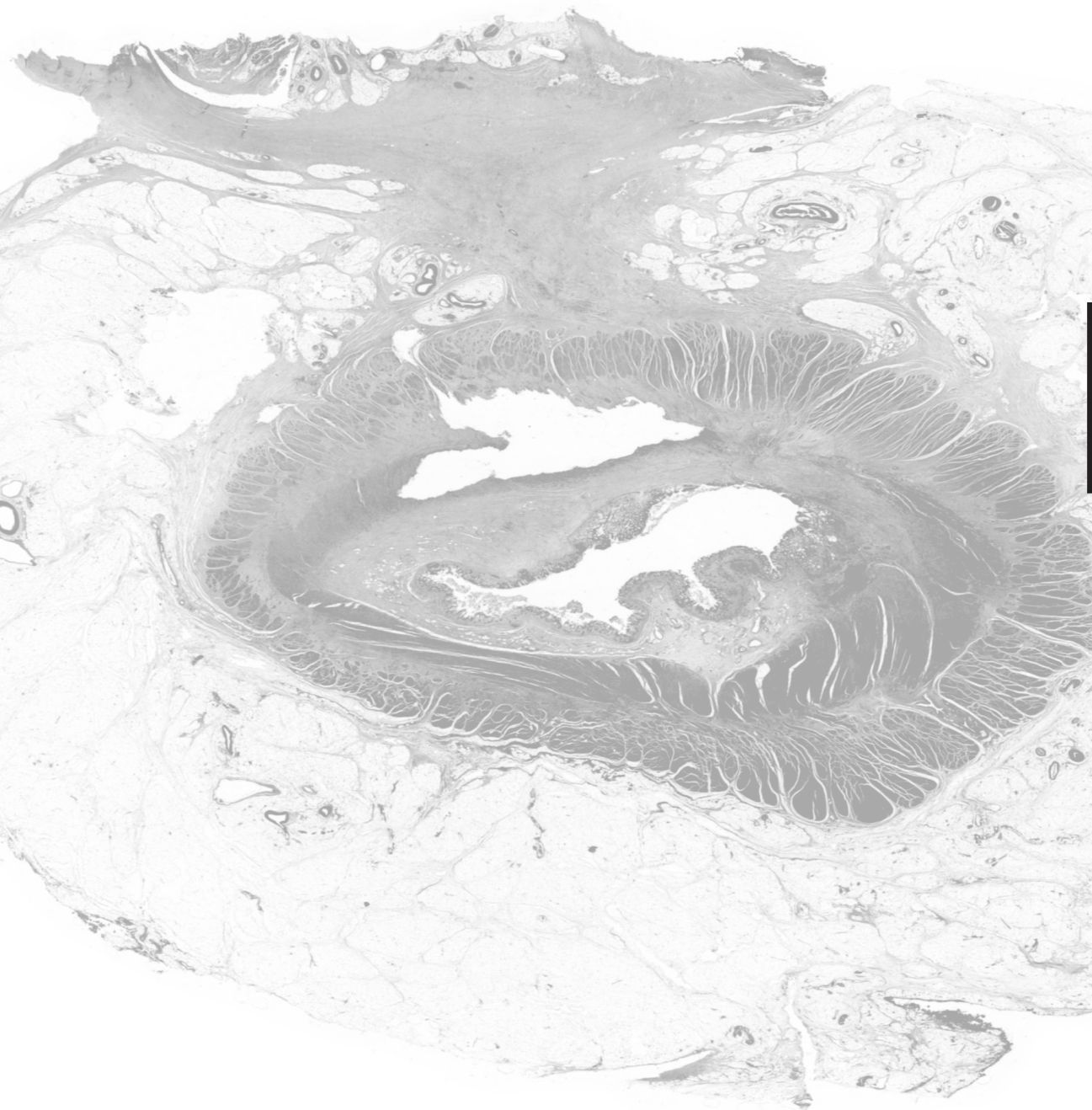

6

PIK3CA kinase domain mutation identifies a subgroup of stage III colon cancer with poor prognosis

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Abstract

PIK3CA mutations in the helical domain (exon 9) and in the kinase domain (exon 20) cause tumor formation by different means. We aimed to determine the effects of each of these mutations on survival of colon carcinoma patients.

A large cohort of 685 colon carcinoma patients was tested for *PIK3CA* mutations in exons 9 and 20 by single nucleotide primer extension (N=428) or by real time PCR (N=257).

PIK3CA mutation rate was 13%. 66 of 83 (79.5%) were in exon 9 and 17 of 83 (20.5%) in exon 20. In survival analysis, *PIK3CA* mutations in exon 9 and 20 had different effects on patient outcome. The *PIK3CA* exon 20 mutation conferred a poorer disease free survival compared to patients with wild type alleles and exon 9 mutations (Log rank p=0.04 and p=0.03 respectively) and cancer specific survival (Log rank p=0.03 and p=0.056 respectively) in stage III patients. In stage I and II this negative effect on outcome was not seen.

PIK3CA mutation in exon 20 is a negative prognostic factor in stage III colon cancer patients. Moreover, this negative effect is not present in stage I and II patients.

Introduction

Tumor classification according to the UICC's or AJCC's TNM classification is the most important prognostic factor in colorectal cancer. According to treatment guidelines, adjuvant chemotherapy is given to patients with stage III disease and with high risk stage II disease. High risk stage II is defined as T4 tumors, clinical presentation as bowel obstruction or perforation, poor differentiation or lymph node yield of less than ten. In these stages, adjuvant chemotherapy improves significantly 5 years survival rates[1,2]. One could argue, though, that some stage III patients could be considered cured after surgery alone and thus do not benefit from the adjuvant chemotherapy and are therefore overtreated. Opposed to the 15% of the node negative stage II patients that do relapse, as this group would probably benefit from adjuvant chemotherapy and are therefore undertreated [2]. To identify these patients, accurate markers of disease prognosis are needed. Over the last decade, several informative molecular prognostic and/or predictive markers have been identified in stage II and III colon cancer patients, such as microsatellite instability (MSI) and chromosome 18q deletion [3-8]. Recently, the *BRAF* V600E mutation was also defined as an important prognostic factor in these patients [9,10].

This search for molecular markers has led to study the mutational status of proteins involved in cellular transduction pathways signaling for cell survival and proliferation. An important protein involved in many cellular functions such as cell proliferation, growth and apoptosis is the phosphatidyl-inositol 3 kinase catalytic subunit alpha (PIK3CA), also known as p110α [11-13]. PIK3CA is an activating protein kinase, which phosphorylates PIP2 into PIP3 facilitating the activation of AKT and further downstream signaling to activate mTOR. PIK3CA forms an heterodimer with its regulatory subunit p85α, which stabilizes PIK3CA and inhibits its kinase activity [13]. Nevertheless, binding with p85α is mandatory for *PIK3CA* activation. PIK3CA is frequently mutated in several malignancies like thyroid, mama, colon and pancreas cancer [14]. Mutated PIK3CA has been found to be oncogenic [15] and to promote disease progression and metastasis in colon cancer models [16]. Mutation frequencies in colon carcinoma vary from 16 to 37%[14,17-19]. The most frequent mutations in the *PIK3CA* gene occur in codons 542 and 545 in exon 9 coding for the helical domain and in codon 1047 in exon 20 coding for the kinase domain. These mutations all induce a gain of function of PIK3CA but they drive cancer progression through different pathways [20]. To induce transformation exon 20

mutants depend on binding with the regulatory subunit p85α whereas exon 9 mutants circumvent p85α binding but depend on RAS binding instead [13,21,22].

Moreover, these mutations could represent phenotypically different histological types of cancer. In breast cancer, for instance, exon 9 mutations were significantly associated with lobular carcinomas [23]. Besides, *PIK3CA* exon 20 mutations were found almost exclusively in hereditary colon carcinoma forms such as Lynch syndrome and familial adenomatous polyposis (FAP); whereas in sporadic forms, exon 9 mutations were significantly more frequent [24].

In colon carcinoma, the prognostic value of *PIK3CA* mutations is controversial. Several authors have reported a negative prognostic effect of *PIK3CA* mutations [18,25] whereas others are unable to reproduce these data [26]. However, till recently mutations have not been studied independently. Therefore, the aim of the present study was to determine the impact of the mutations either present in the helical or in the kinase domain of *PIK3CA* on survival of colon cancer patients.

PATIENTS AND METHODS

Patients

Six hundred and eighty five patients diagnosed with stage I (n=49), stage II (n=223) and stage III (n=413) colon carcinoma between 1990 and 2006 were included in this study. The majority of the patients (N=456, 67%) were diagnosed between 1997 and 2004, 166 (24%) were diagnosed prior to 1997 and 63 (9%) between 2004 and 2006. Four hundred twenty eight patients (62.5%) were diagnosed at the PAMM laboratory for Pathology and treated in four different hospitals in the Eindhoven region in the south of the Netherlands. Whereas two hundred fifty seven patients (37.5%) were diagnosed and treated at the Leiden University Medical Centre (LUMC), Leiden, the Netherlands. The 428 patients from Eindhoven are hereafter called the PAMM cohort and the 257 patients from Leiden, the LUMC cohort.

Of the stage II patients, 131 (58%) would nowadays be considered as high risk stage II mostly due to the insufficient number of lymph nodes examined at the time of diagnosis and would therefore receive adjuvant chemotherapy. From these high risk patients only 6 (4%) were treated with adjuvant chemotherapy as well as four patients with stage II disease. Of the stage III patients, 296 received adjuvant chemotherapy. In total, 306 patients (45%) received adjuvant chemotherapy after surgery whereas 371 (54%) did not. Data from eight patients on adjuvant chemotherapy was missing (1%).

A tumor was considered right sided when it was located between the coecum and the splenic flexure. A left sided tumor was located between the splenic flexure and the rectosigmoid. Rectum tumors were excluded from the study since these patients are treated differently from colon cancer patients.

Demographic data were obtained from the database of the Eindhoven Cancer Registry maintained by the Comprehensive Cancer Centre South. Follow-up data were collected from the medical records and the Oncdoc registration at the LUMC.

Patient's characteristics of the entire group are depicted in Table 1. Briefly, median age was 68 years (22-94), 53% (N=362) was male, and 53% (N=352) had a right sided tumor. The majority of the patients had a T3 (N=481; 71%), well or moderately differentiated (N=456; 76%) and microsatellite stable tumor (N=498 82%). Median follow up was 49 months (0-219) and median time to progression was 44 months. At the end of the follow up period, 49% of the patients were still alive without evidence of disease, 7% was alive with disease whereas 24% had died because of cancer related causes and

20% had died because of causes other than cancer as specified in their medical records. The use of clinical material for this retrospective study was approved by the institutional review boards according to the guidelines of the Dutch Federation of Medical Research Associations (www.fmww.nl viewed January 2009).

Tumor tissue was selected by experienced pathologists (GvL, HM) using diagnostic hematoxyline-eosine (HE) slides. Tumor cell percentages of at least 30% tumor cell were set. This cut-off value is higher than the analytic detection limits of both techniques used. Subsequently, selected areas were macrodissected or punched with a 2mm core needle. DNA was isolated after proteinase K digestion of formalin fixed paraffin embedded tissue (FFPE) and subsequent purification with either HPTTP kit (Roche, Almere, the Netherlands) or Nucleospin Tissue (Macherey Nagel, Düren, Germany) following manufacturer's instructions with minor modifications.

Mutation analysis

In the PAMM cohort, *PIK3CA* mutations were determined by PCR followed by single nucleotide primer extension assay, as described by Hurst et al. [27] for the hotspots in exon 9, c.1624G>A; p.E542K and c.1633G>A; p.E545K and in exon 20 the c.3140A>G; p.H1047R. Briefly, both exons were amplified by multiplex PCR. After enzymatic purification of the PCR products with EXO SAP (USB Co, Staufen, Germany), the extension reaction was performed using primers published elsewhere [27] and the ABI Prism® SNaPshot™ multiplex kit (Applied Biosystems, Nieuwerkerk aan den IJssel, the Netherlands). Finally, these products were purified and separated by capillary electrophoresis using an ABI 3130 (Applied Biosystems, Nieuwerkerk aan den IJssel, the Netherlands).

In the LUMC cohort, *PIK3CA* mutation detection of the same hotspots was performed by real time PCR enabling allelic discrimination using primers and probes designed and ordered by Applied Biosystems and TAQMAN chemistry (Applied Biosystems, Nieuwerkerk aan den IJssel, the Netherlands). Assays were performed in a Roche Light Cycler 480 (Roche, Almere, the Netherlands) (manuscript in preparation).

In 69 (10%) samples, mutations could not be typed due to poor DNA quality after isolation. However, missing data were equally distributed between groups and was not a source of bias (see table 1).

Sensitivity was not an issue in this study, as all of the samples tested contained more than 30% tumor cells and the detection threshold of both techniques ranges between

1-10% tumor cells. To test specificity and sensitivity, several samples were tested with both techniques and compared with direct sequencing. No discrepancies were found.
The existence of a putative pseudogene on chromosome 22 overlapping exon 9 to 13 of the *PIK3CA* gene does not influence our results because the mutations studied here correspond to E545K and E542K which are not present in the pseudogene.
In the PAMM cohort, MMR status, mutations in *BRAF* V600E and *KRAS* codons 12 and 13 had been previously determined and described [10].
In the LUMC cohort MMR status was determined with the multiplex kit, MSI Analysis System version 1.2. (Promega, Madison, WI, USA). Products were separated by capillary electrophoresis using an ABI 3130 (Applied Biosystems, Nieuwerkerk aan den Ijssel, the Netherlands).

Statistical analyses

All statistical analyses were performed using SPSS version 16 software for Windows (Chicago, Il, USA).
To study associations between categorical variables χ^2 test and Fisher's exact test were used. Associations between categorical and continuous variables were studied by ANOVA. Univariate and multivariate survival analyses were performed by Kaplan Meier and Cox Proportional Hazards method. Survival curves were compared using the Log Rank method.
Disease Free Survival (DFS) is defined as the time between surgery and disease progression being the development of distant metastasis or local recurrence or death due to cancer related causes whatever happened first. Cancer Specific Survival (CSS) is the period of time between surgery and death because of cancer related causes. Overall survival (OS) is defined as the period of time between surgery and death because of any cause.

RESULTS

Mutation analysis

PIK3CA mutations were found in 83 (13%) patients. In the majority of the patients (N=66; 79.5%) the mutation was located in exon 9, the helical domain coding region and in 17 (20.5%) cases a mutation was detected in exon 20, the kinase domain coding region. No double mutants were found in the whole cohort.

In the present cohort of patients *PIK3CA* mutations were associated with MMR status. MSI-H tumors had significantly more frequently a *PIK3CA* mutation in exon 20 (p=0.006) (Table 1).

The frequencies of the additionally typed mutations in the PAMM cohort were as follows: 34% *KRAS* mutations in codons 12 and 13 and 21% *BRAF* V600E mutation as previously described [10]. There were no significant associations found between these mutations and *PIK3CA* mutations (Table 2).

Survival analyses

Univariate analysis

All *PIK3CA* mutations together did not influence survival in the present group of patients. OS, DFS and CSS did not differ between wild type and mutant patients.

However, the effect of each of the *PIK3CA* mutations on CSS and DFS differed. *PIK3CA* exon 9 mutations did not affect survival in the studied disease stages, whereas *PIK3CA* exon 20 mutations conferred a poorer DFS and CSS to stage III patients only (DFS Log Rank wt vs. exon 20 p=0.04 and exon 9 mutation vs. exon 20 p=0.03; CSS Log Rank wt vs. exon 20 mutation p=0.03 and exon 9 mutations vs. exon 20 mutation p=0.056). This negative effect in patient's outcome of the latter mutation was not seen in stage I and II. As the survival curves from stage I and stage II did not differ, both stages were grouped for the survival analysis and were compared with stage III tumors (CSS Kaplan Meier plot is shown in Figure 1). The lack of negative effects in survival of the exon 20 mutation in stage I and II disease was maintained in the high risk stage II group (Figure 2). No effect of exon 20 mutations in OS was seen.

Table 1: Patient's characteristics and association with *PIK3CA* mutations.

Variables	Total N (%)	Total patients analysed for *PIK3CA* mutations * N (%)	*PIK3CA* wt N (%)	*PIK3CA* mut ex 9 N (%)	*PIK3CA* mut ex 20 N (%)
		616	533 (87)	66 (10)	17 (3)
Gender					
Male	362 (53)	327 (53)	279 (85)	36 (11)	12 (4)
Female	322 (47)	288 (47)	253 (88)	30 (10)	5 (2)
Tumor location					
Right	352 (53)	323 (54)	277 (86)	34 (10)	12 (4)
Left	313 (47)	278 (46)	243 (87)	30 (11)	5 (2)
Stage					
I	49 (7)	35 (6)	29 (82)	3 (9)	3 (9)
II	223 (33)	194 (31)	167 (86)	20 (10)	7 (4)
III	413 (60)	387 (63)	337 (87)	43 (11)	7 (2)
Stage II High Risk	131 (19)	111 (18)	94 (85)	12 (11)	5 (4.5)
N stage					
N0	260 (38)	229 (37)	196 (86)	23 (10)	10 (4)
N+	408 (62)	387 (63)	337 (87)	43 (11)	7 (2)
T stage					
T1	22 (3)	16 (3)	13 (81)	1 (6)	2(13)
T2	66 (10)	52 (8)	45 (86)	5 (10)	2 (4)
T3	481 (71)	443 (72)	389 (88)	44 (10)	10 (2)
T4	112 (16)	102 (17)	83 (81)	16 (16)	3 (3)
Differentiation grade					
Well/moderately	456 (76)	422 (77)	360 (85)	49 (12)	13 (3)
Poor/Undifferentiated	145 (24)	124 (23)	111 (90)	9 (7)	4 (3)
MMR status					
MSS	498 (82)	466 (82)	407 (87)	52 (11)	7 (2)
MSI-H	107 (18)	102 (18)	86 (84)	9 (9)	7 (7)#
Median age (min-max)	68 (22-94)	68 (30-94)	68 (30-93)	67 (35-94)	67 (45-84)
Median Follow-up in months (min-max)	49 (0-219)	49 (0 – 219)	49 (0-219)	47.5 (0-177)	48 (9-124)
Disease Progression					
No progression	459 (69)	420 (70)	363 (86)	47 (11)	10 (3)
Progression	208 (31)	183 (30)	159 (87)	18 (10)	6 (3)

* It was not possible to perform DNA mutation analysis in 10% of the patients as described in materials and methods.
#p=0.006

Multivariate analysis: Cox Proportional Hazards model

In a multivariate survival analysis adjusting for age, gender, tumor location, adjuvant chemotherapy, T stage, MMR status and tumor differentiation, *PIK3CA* mutations in exon 20 were significant negative prognostic factors in stage III tumors (CSS HR 4.53 95% CI 1.56 – 13.2 p=0.006), whereas in stage I and II tumors no significant effect was seen (Table 3). *PIK3CA* exon 9 mutations did not affect survival neither in the multivariate analysis.

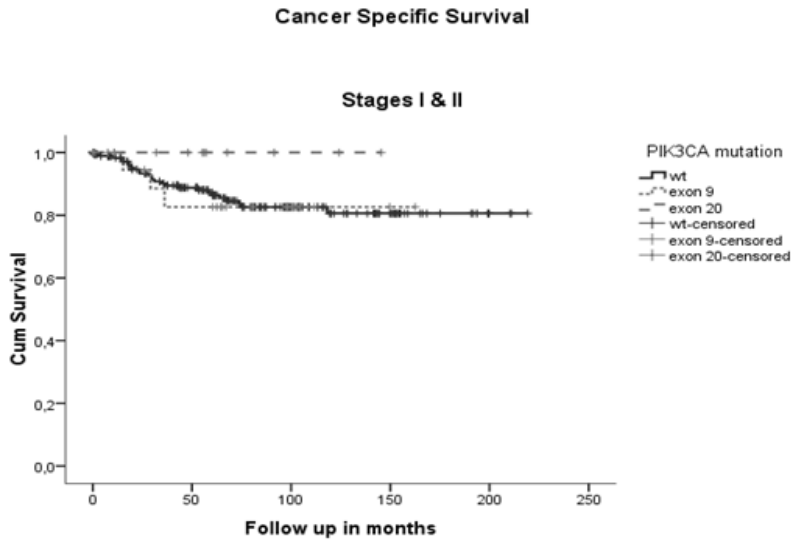
In a multivariate survival analysis in the PAMM cohort adjusting for the above mentioned prognostic variables and adding *BRAF* V600E mutation, *KRAS* codon 12 and 13 mutation, the negative effects of the *PIK3CA* exon 20 mutation on DFS, CSS and OS remained significant in the stage III group. In the stage I and II group, *PIK3CA* exon 20 had no significant effect on survival (data not shown).

Table 2: *KRAS* and *BRAF* mutations frequencies and associations with *PIK3CA* mutations in the PAMM cohort.

	Total N (%)	Total analysed for *PIK3CA* mutations §	*PIK3CA* wt	*PIK3CA* mut ex 9	*PIK3CA* mut ex 20
KRAS					
wt	276 (66)	270 (67)	234 (87)	28 (10)	8 (3)
mut	143 (34)	133 (33)	111 (84)	19 (14)	3 (2)
BRAF					
wt	336 (79)	320 (78)	276 (86)	37 (12)	7 (2)
V600E mut	89 (21)	88 (22)	71 (81)	13 (15)	4 (4)

§ It was not possible to perform DNA mutation analysis in 10% of the patients as described in materials and methods.

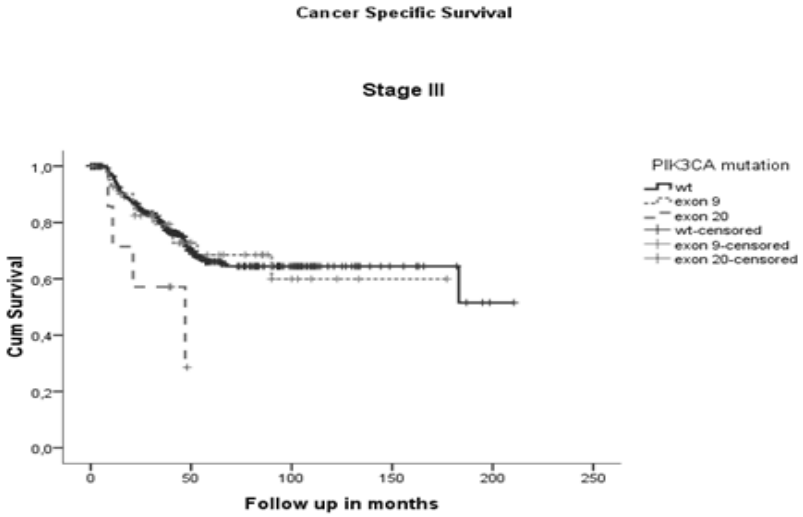
a) Stage I and II



wt N=186 exon 9 N=23 exon 20 N=9

Log Rank wt vs. exon9 p=0.93; wt vs. exon20 p=0.24; exon9 vs.exon20 p=0.21

b) Stage III

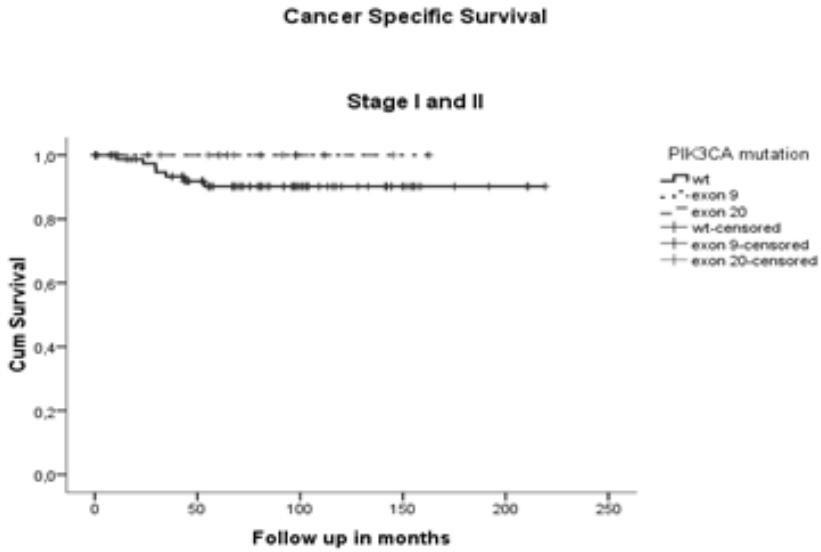


wt N=326 exon 9 N=42 exon 20 N=7

Log Rank wt *vs.* exon9 p=0.94; wt *vs.* exon 20 p=0.029; exon 9 *vs.* exon 20 p=0.056

Figure 1: Kaplan Meier plots in a)stage I and stage II and b) stage III disease for Cancer Specific Survival according to *PIK3CA* mutations.

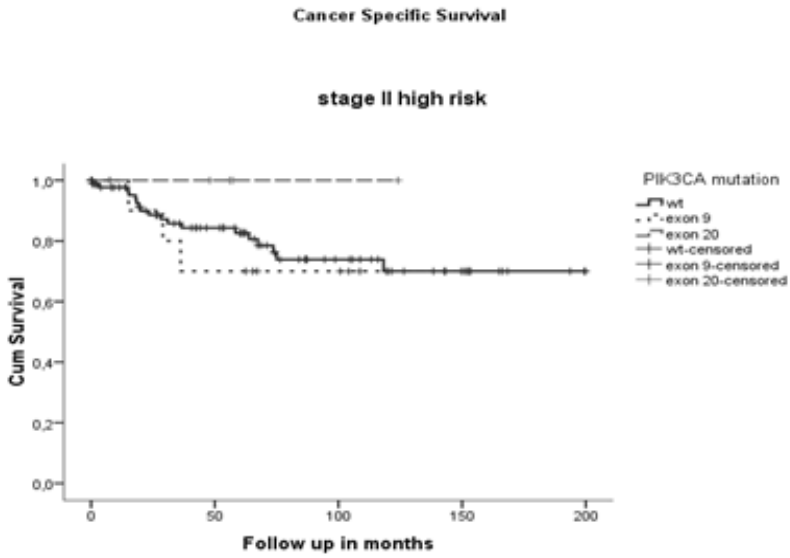
a) Stage I and II



wt N=83 exon 9 N=8 exon 20 N=5

Log rank wt ex9 p=0.4, wt ex20 p=0.5

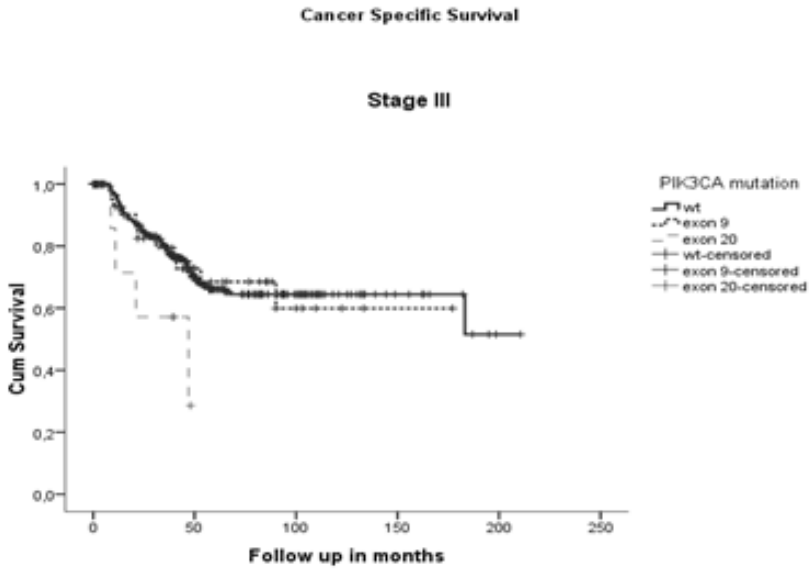
b) Stage II High Risk



wt N=91 exon 9 N=12 exon 20 N=4

Log rank wt ex9 p=0.6, wt ex20 p=0.3, ex9 ex20 p=0.25

c) Stage III



wt N=326 exon 9 N=42 exon 20 N=7
Log rank wt ex9 p=0.9, wt ex20 p=0.03, ex9 ex20 p=0.06

Figure 2: Kaplan Meier plots for Cancer Specific Survival in a) stage I and II, b) high risk stage II and c)stage III according to *PIK3CA* mutation.

Table 3: Cox proportional hazard model for CSS in the whole group.

Overall Survival	Variables in the model	HR	p-value	95% CI
Stage I and II (N=149)	***PIK3CA***			
	PIK3CA wt	referent		
	PIK3CA exon 9	0.86	0.85	0.19 - 4
	PIK3CA exon 20	0	0.91	0– 2.2E68
	T status			
	T2	referent		
	T3	13266.33	0.95	0 – 1.2E119
	T4	63038.4	0.94	0 – 1.4E120
	Category differentiation			
	Well/moderately diff.	referent		
	Poor/Undiff.	1.52	0.57	0.36 – 6.34
	Adjuvant chemotherapy			
	No	referent		
	Yes	3.09	0.24	0.48 – 20.08
	Age	1.02	0.34	0.98 – 1.07
	Gender			
	Male	referent		
	Female	1.62	0.32	0.63 – 4.1
	Tumor Location			
	Right sided	referent		
	Left sided	0.91	0.87	0.3 – 2.78
	MMR status			
	MSS	referent		
	MSI-H	0.91	0.89	0.22 – 3.73
Stage III (N=305)	***PIK3CA***			
	PIK3CA wt	referent		
	PIK3CA exon 9	1.11	0.77	0.55 – 2.22
	PIK3CA exon 20	**4.53**	**0.006**	**1.56 – 13.2**
	T status			
	T2	**referent**		
	T3	**0.042**	**0.002**	**0.005 – 0.33**
	T4	**0.50**	**0.005**	**0.31 – 0.81**
	Category differentiation			
	Well/moderately diff.	**referent**		
	Poor/Undiff.	**1.6**	**0.05**	**0.99 – 2.74**
	Adjuvant chemotherapy			
	no	referent		
	yes	0.68	0.19	0.38 – 1.21
	Age	1.001	0.91	0.98 – 1.03
	Gender			
	Male	referent		
	Female	0.98	0.92	0.63 – 1.52
	Tumor Location			
	Right sided	referent		
	Left sided	1.16	0.54	0.73 – 1.83
	MMR status			
	MSS	referent		
	MSI-H	0.73	0.35	0.38 – 1.41

Results in **bold** are statistically significant.

Discussion

Hotspot mutations in *PIK3CA* causing the constitutive activation of the protein, contribute to cell transformation and tumor progression. Mutations in the helical and the kinase domain cause cellular transformation and tumor progression by different means[20,21,23,28-32]. Until recently, the impact on colon cancer survival of these different mutations has not been studied.

To study the effects of the different mutations separately, a large cohort of patients was mandatory. This need implied collecting material through a relatively broad period of time i.e. 1990-2006. During this period, colon cancer treatment protocols and guidelines changed improving survival. However, the improvement in survival trends for stage II disease took place before 1995 and for stage III survival improvement was mainly due to the introduction of adjuvant chemotherapy in the mid nineties [33,34]. Therefore, this issue does not influence the results as the majority of the patients studied were diagnosed after 1995 and administration of chemotherapy was one of the variables corrected for in the multivariate model.

In the present cohort of colon carcinoma patients, the *PIK3CA* mutations in exon 9 and in exon 20 had different effects on survival. Mutations in exon 9 did not affect survival, whereas exon 20 mutations had a negative effect on survival but only in stage III patients. Only recently, the prognostic value of *PIK3CA* mutations in colon cancer was studied segregating both mutation types. De Roock and colleagues [35] published an interesting report over several gene mutations involved in resistance to anti-EGFR therapy in metastatic colorectal carcinoma. These authors concluded that only *PIK3CA* exon 20 mutations influenced survival of a group of patients with metastatic colorectal cancer chemotherapy refractory treated with chemotherapy and cetuximab. Our findings support the theory of a different effect of exon 20 and exon 9 mutations on survival. The data of de Roock *et al* together with the present data could explain the inconclusive results previously published on colon cancer survival with respect to the effect of *PIK3CA* mutations[18,25,26].

Furthermore, in the present study we described what might be a stage dependent survival effect, as mutations in exon 20 conferred a poor survival but only in stage III disease whereas this deleterious effect was not present in stage I/II patients with *PIK3CA* exon 20 mutations. This trend was maintained in the stage II high risk group.

As it could be expected based on the described association between exon 20 mutations

and Lynch syndrome [24], mutations in the kinase domain were significantly more frequent in MSI-H tumors. An interesting question is whether microsatellite instability confounds the results on outcome of patients with exon 20 mutations. Microsatellite instability confers a better prognosis to stage II and to stage III if not treated with 5-FU [3,8]. From our analyses it can be concluded that the prognostic effects of exon 20 mutations are independent of the microsatellite status of the tumor as survival effects of these mutations were seen in both groups of patients MSI-H and MSS.

The biological background of this stage dependent prognostic effect could be explained by the fact that the *PIK3CA* protein mutated in its kinase domain must necessarily bind to its regulatory subunit p85α in order to have transforming capacities [13,22]. Although, p85α is not frequently found mutated in human cancer, its expression is altered. Indeed, p85α is differentially expressed in adenoma tissue compared to carcinomas as shown by immunohistochemistry in colonic tissue. Moreover, expression of p85α increases as well with disease stage [36] and is highly overexpressed in node positive tumors [37] whereas *PIK3CA* exon 20 mutations frequency do not differ significantly among different stages. Thus, it could be hypothesized that through the low levels of expression of the regulatory subunit in stage I and II tumors, exon 20 *PIK3CA* might not have enhanced tumorigenic capacity and hence the better survival of this group of patients, whereas in stage III tumors, tumorigenic capacity would be present resulting in a poorer outcome. This hypothesis is currently under investigation.

This study has the limitations inherent to its retrospective character and to the frequency of exon 20 mutations; therefore validation of these data in a larger retrospective cohort or preferably in a prospective study is necessary in order to confirm the potentially relevant clinical consequences of this study.

Targeted therapies inhibiting *PIK3CA* signaling are currently under investigation in clinical trials [38]. However, if our results are confirmed, individual *PIK3CA* mutations should be assessed and correlated to disease stage since patients with early stages disease carrying *PIK3CA* exon 20 mutations seemed to have a better natural history of their disease and further treatment might be unnecessary. On the other hand, stage III patients with *PIK3CA* exon 20 mutations would probably benefit from additional targeted therapies.

In conclusion, the prognostic value of mutations in the *PIK3CA* gene is different according to the type of mutation. Mutations in exon 9 do not influence outcome whereas exon 20 mutation has a prognostic impact among patients with stage III colon cancer. In

stage I and II patients however, the same mutation did not have any negative effect on survival. Moreover, the lack of deleterious effects on outcome is also present in a high risk stage II patient's population and might therefore harbor true clinical implications. In future studies addressing the mutational status of *PIK3CA* both hotspots should be analyzed separately.

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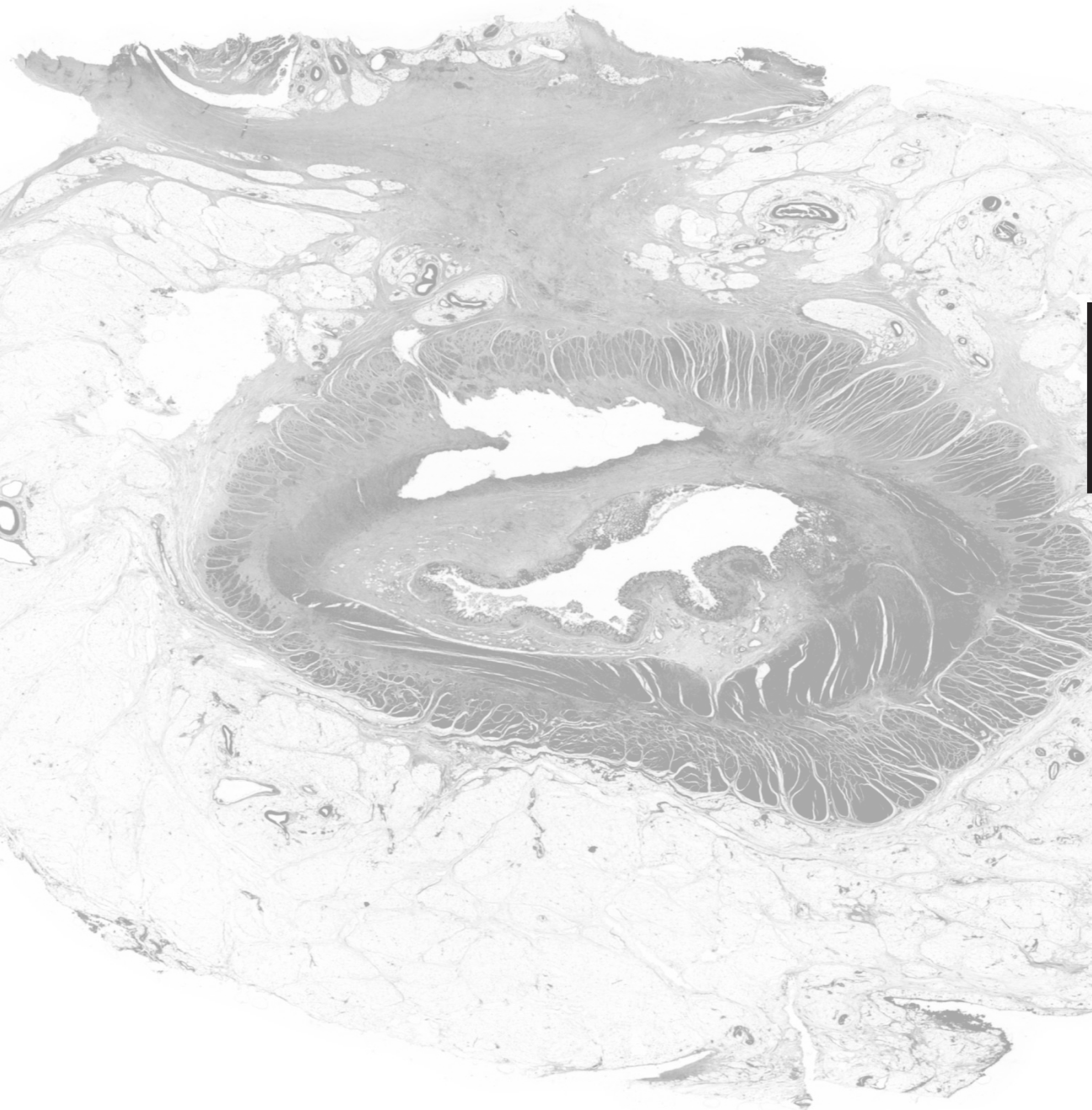

7

CSNK1A1 expression modifies *TP53* effects on survival of colon cancer patients

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Abstract

p53 (encoded by *TP53*) is involved in DNA damage repair, cell cycle regulation, apoptosis, aging and cellular senescence. *TP53* is mutated in around 50% of human cancers. Nevertheless, the consequences of p53 inactivation in colon cancer outcome remain unclear. Recently, the role of p53 together with CSNK1A1 in colon cancer invasiveness has been described in mice.

By combining data on different levels of p53 inactivation, we aimed to predict p53 functionality and to determine its effects on colon cancer outcome. Moreover, survival effects of *CSNK1A1* together with p53 were also studied.

Eighty-three formalin fixed paraffin embedded colon tumors were enriched for tumor cells using flow sorting, the extracted DNA was used in a custom SNP array to determine chr17p13-11 allelic state; p53 immunostaining, *TP53* exons 5, 6, 7 and 8 mutations were determined in combination with mRNA expression analysis on frozen tissue.

Patients with a predicted functional p53 had a better prognosis than patients with non functional p53 (Log Rank p=0.009). Expression of *CSNK1A1* modified p53 survival effects. Patients with low *CSNK1A1* expression and non-functional p53 had a very poor survival both in the univariate (Log Rank p<0.001) and in the multivariate survival analysis (HR=4.74 95% CI 1.45 – 15.3 p=0.009).

In conclusion, the combination of genetic, genomic, protein and downstream transcriptional activity data is very informative of p53 functionality. The predicted p53 functionality has a prognostic effect on colon cancer patients. This effect was modified by *CSKN1A1* expression.

Introduction

During colon carcinogenesis cells accumulate several genetic and genomic aberrations that lead to uncontrolled proliferation and tumor formation [1]. A major event in the adenoma to carcinoma transition is *TP53* inactivation. p53 plays a crucial role in maintaining genome stability and integrity. Upon DNA damage, the activation of p53 leads to cell cycle arrest enabling the cells to repair the damaged DNA. On the other hand, when the damage is too extensive to be repaired p53 activation can also drive the cell towards apoptosis or senescence [2]. Recently, p53 has also been implicated in tumor invasiveness [3]. In mice, the inactivation of casein kinase 1 alpha (Csnk1a1) promotes the cytoplasmatic/nuclear accumulation of β-catenin which stimulates the transcription of Wnt signaling target genes. The combined inactivation of p53 and Csnk1a1 rapidly leads to tumor invasiveness in the colon of these mice.

Inactivation of *TP53* is one of the most frequent events in human cancer [4]. Among others, *TP53* can be inactivated by "loss of function" mutations in one allele and deletion of the remaining wild type allele or by dominant negative mutations that are able to inactivate also the wild type protein transcribed by the second unaffected allele. Either way, when p53 function is jeopardized, genomic instability and uncontrolled cell proliferation are facilitated.

The role of p53 inactivation in colon cancer progression and prognosis has been widely studied but remains elusive notwithstanding the amount of reports addressing this subject [5-17]. Chromosomal instability (CIN) is a known prognostic factor in colon cancer[18]. Although *TP53* inactivation has been frequently associated with CIN, not all tumors with CIN carry an inactive p53 and vice versa [19]. More complexity is added by the recent demonstration that *TP53* can behave as a haploinsufficient tumor suppressor gene. Using mouse models, Ventakachalam and coworkers demonstrated that mice carrying one functional p53 allele developed tumors but they showed however a milder phenotype than mice that lost both alleles [20]. Moreover, several reports described the *TP53* gene dosage effect on expression of target genes [21, 22].

Recent developments in genomic copy number analysis have shown to more accurately study the measure of chromosomal structural and numeric aberrations [23]. The development of the lesser allele intensity ratio (LAIR) algorithm that integrates the DNA index in the analysis of copy number data gives a real measure of the chromosomal alterations and allows the study of gene dosage effects in tumors.

Given the complexity of the p53 network, the several ways of p53 inactivation, and the recently described role of p53 in cancer invasiveness in mice, we studied in detail different levels of p53 inactivation in human colon cancer taking into account the allelic state of the locus on the short arm of chromosome 17, gene mutation state, protein expression levels, downstream target gene expression and determine the prognostic impact in colon cancer patients. Moreover, interactions with the recently described *CSNK1A1* expression and the impact on disease outcome were also explored.

Patients and Methods

Patients

Eighty three colorectal cancer patients diagnosed as stage I, II or III at the Leiden University Medical Centre between 1991 and 2005 were selected for the present study.

Methods

Tissue preparation for multiparameter flow cytometry and sorting

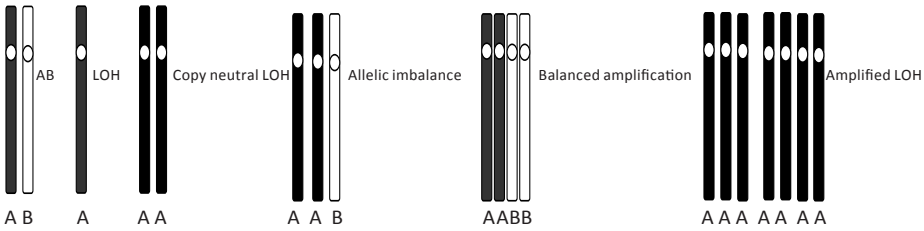
Tumor and stromal cells were sorted from FFPE tissue blocks using the FACS ARIA I (BD Biosciences, San Jose, CA, USA) based on vimentin, keratin and DNA content as previously described by Corver *et al* [24, 25]. DNA index (DI) defined as the ratio between the median G0/G1 keratin fraction and the median G0/G1 vimentin fraction, was calculated using a remote link between Winlist and ModFit (Verity Software House) for each sample. Whenever more than one keratin positive clone was seen, it was independently sorted. DI was categorized as DI< 0.95; DI=0.95 – 1.05; DI=1.06 – 1.4; DI=1.41 – 1.95 and DI>1.95.

DNA was purified from sorted cells after an overnight proteinase K digestion using the Nucleospin Tissue kit (Macherey Nagel, Düren, Germany) following manufacturer's instructions.

SNP array hybridization for allelic state determination

A custom Golden Gate genotyping panel with 384 SNPs was designed using the Assay Design Tool (Illumina Inc. San Diego, CA, USA). The panel contains SNPs mapping the the following chromosomes: 1q21-25, 8q22-24, 13q12-34, 17p13-11 (the *TP53* locus), 18q12-22 and 20q11-13, all of which are associated with tumor progression in the colorectum [26]. SNPs on chromosome 2 serve as controls. Paired samples were analysed in the Golden Gate assay as described [27] and hybridized to Sentrix Array Matrix with 384 bead types. SNP arrays were analysed in the BeadarraySNP package. The data generated was analyzed with the LAIR algorithm [23] that integrates the DNA index into the analysis. We differentiated the following allelic states: 1) genotype AB or normal; 2) genotype A or loss of heterozygosity (LOH); 3) copy neutral LOH (CN LOH) or genotype AA; 4) amplified LOH (amp LOH) corresponding to genotype AAA or AAAA; 5) allelic imbalance (AI) or genotype AAB, AAABB; 6) balanced amplification (BA) corresponding

a)



b)

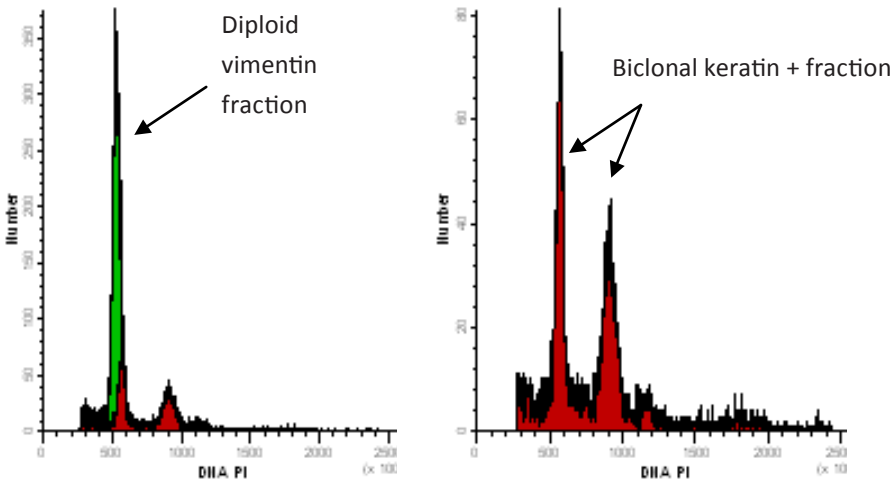


Figure 1:

a) Schematic representation of the possible allelic states according to LAIR scores

b) Example of a DNA histogram of one tumor containing two clones with different DNA indexes. Green histogram is the diploid vimentin fraction and in red the keratin fraction.

to AABB genotypes and finally multiclonal samples whenever more than one clone was seen by flow cytometry (Figure 1)[23].

FISH

To confirm the copy number results obtained with the SNP array, FISH in nuclei obtained from FFPE material of seven patients was performed. First, 2mm. punches (Beecher Instruments, Silver Springs, MD, USA) of selected tumor areas were embedded in blanco acceptor paraffin blocks. Subsequently, 50 µM slices were obtained, deparaffinized and rehydrated. Antigen retrieval was performed by high pressure cooking in Tris-EDTA pH=9. After incubation for one hour at 37°C with RNAse, samples were digested with 0.5% pepsin pH=2 at 37°C for 30 minutes. The obtained nuclei were then washed and resuspended in methanol: acetic acid in a 3 to 1 proportion. Thereafter nuclei were spun onto clean glasses and hybridization with Vysis® TP53/CEP17 FISH probe kit (Abbot Molecular, IL, USA) was allowed overnight at 37°C. After washing, samples were mounted with Vectashield® mounting medium containing DAPI (Vector Laboratories Inc., Burlingame, CA, USA) and nuclei were evaluated under the fluorescence microscope.

Seven patients were tested from whom enough material was available and with different allelic states of chr.17p according to the SNP array analysis.

p53 IHC staining

Tissue microarrays (TMA) of these tumors were prepared by punching three representative tumor areas selected by a pathologist (HM) on HE stained slides and arraying them on a recipient paraffin block (Beecher Instruments, Silver Springs, MD, USA). Five µM slices were then cut. Heat induced antigen retrieval (HIAR) was performed as described elsewhere [26] and staining was carried out with the mouse anti-human monoclonal antibodies directed against p53 (clone D0-7, 1:1000 dilution) (Lab Vision NeoMarkers, Fremont, CA, USA).

p53 was scored in four different categories based on any level of nuclear staining: completely negative; 1- 25% positive nuclei (indicative of a wild type state); 25-75% positive nuclei and >75% positive nuclei. For analysis purposes, the last two categories were fused in only one category; more than 25% positive cells (indicative of a mutated gene).

TP53 mutation analysis

Tumor DNA was isolated from enriched tumor areas containing at least 50% tumor cells by proteinase K digestion followed by purification with Nucleospin Tissue kit (Marcherey Nagel, Düren, Germany). DNA was available from 40 patients. Four different PCRs were performed for amplification of exons 5, 6, 7 and 8 of the TP53 gene. Ten nanograms DNA were used for each PCR using primers already published modified for SYBRgreen® detection [28]. Subsequently, PCR products were purified using Qiagen's MinElute™96 UF PCR Purification Kit (Qiagen Sciences, Germantown, MD, USA) and reactions were sequenced using the MI13 forward and reverse primers. Analysis was performed using the Mutation Surveyor 3.97® sequence analysis and assembly software (SoftGenetics LLC, Stage College, PA, USA).

mRNA expression arrays

Fresh frozen tissue of fifty seven patients was available for mRNA expression analysis. mRNA was isolated, labeled and hybridized to customized Agendia 44 K oligonucleotide array as described elsewhere [29]. The expression of the 35 genes reported by Yoon *et al*[22] as genes which expression is *TP53* gene dosage dependent was analyzed in relation with p53 functional state. Furthermore, expression levels of three probes targeting different locations in the 3'UTR of the CSNK1A1 gene (NM_001025105.1 transcript) were independently analyzed.

Finally, expression levels of eight genes reported by Elyada *et al* (3) as involved in murine tumor invasiveness were also analyzed.

Statistical analysis

Associations between categorical variables were studied by χ^2 and Fischer exact test. Univariate survival analysis was performed by Kaplan Meier analysis and differences between survival curves were studied by Log Rank analysis. Multivariate survival analysis was performed by Cox Proportional Hazard Model. Cancer Specific Survival was defined as the time between curative intended surgery and dead by cancer related causes. Results were considered significant when p value <=0.05. All tests were two tailed. All of the analyses mentioned above were performed using SPSSv16 package for Windows (Chicago, Il, USA)

Statistical analysis of the mRNA expression data was done using the LIMMA (Linear Modelling for Microarray Analysis) framework in Bioconductor[30].

Results

Patients' description

Patients' characteristics are shown in table 1. Summarized, 54% of the patients were female, 63% of the tumors were right sided (i.e. tumors located in the colon from the coecum until the splenic flexure) and 37% left sided. 4% of the patients had stage I disease at diagnosis, 61% stage II and 35% stage III. Twenty seven tumors were MSI-H (33%), whereas 55 (67%) were MSS tumors.

Median follow up was 69 months (range 2 – 199). At the end of the follow up, 41% of the patients were alive, 24% of the patients had died because of cancer related causes and 30% died because of non cancer related causes.

Allelic state

All samples were flow cell sorted as previously described and analyzed with a costum SNP array comprising several chromosomal regions previously reported to be implicated in colorectal cancer progression [26]. In the present study we have focused on the allelic state of the *TP53* locus on chromosome 17p13-11. Of the 83 tumors analyzed, 47% were classified as normal with genotype AB, 11% as LOH (genotype A), 13% as CN LOH (genotype AA), 8% as amp LOH (genotype AAA/AAAA) and 4% as AI (genotype AAB/AAABB). Note also that 17% of the patients showed multiple cancer clones by flow cytometry (results shown in table 1). No balanced amplification corresponding to AABB genotypes was seen in the monoclonal series. LAIR scores were determined by four observers independently.

The LAIR scores of four samples randomly selected were confirmed by FISH (figure 2). Three out of 83 samples with discordant LAIR scores between the observers were also assessed by FISH for the definitive categorization.

Predicted p53 functionality

The predicted functionality of p53 (hereafter called functionality) was determined for each sample by combining data from the *TP53* locus allelic state, mutation data and protein expression levels.

Associations between p53 functionality and the different variables are shown in table 2. Summarizing, the majority of functional p53 (78%) had no mutation in *TP53* (p=0.01) and all of them had between 0-25% positive stained cells using immunohistochemistry

(p<0.0001). 78% of the tumors with functional p53 had a near diploid DNA index raging from 0.95-1.05 whereas 63% of the non functional p53 samples was highly aneuploid with DNA indexes ranging 1.41 – 1.95 (p<0.001). Samples with a functional p53 had significantly more frequently the normal AB genotype, than amp LOH (genotype AAA/AAAA) (p=0.005), CN LOH (genotype AA) (p<0.001) and than tumors with two clones (p=0.006). Moreover, functional p53 was also more frequently seen in the LOH class (genotype A) than in the CN LOH class (genotype AA) (p=0.01). Tumors with a functional p53 were significantly more frequently right sided tumors (p=0.035). Eighty six percent of the tumors with non functional p53 were MSS tumors (p=0.009).

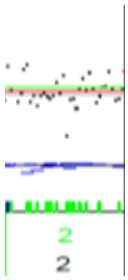
To corroborate the classification in functional and not functional p53, we compared p53 target gene expression levels between these two groups. We selected genes which expression was previously shown to be p53 gene dosage dependent by Yoon *et al* [22]. Eight genes differently expressed between both groups were identified (table 3). As expected, known p53 targets like *MDM2* and *CDKN1A* were higher expressed in the p53 functional group than in the non functional group (p=0.0025 and p=0.0013 respectively). Genes higher expressed in the non functional group were involved in many processes such as cell proliferation (*PRKCZ*), protein ubiquitination (*SIAH1*), metabolism (*HMGCS1*) and cell differentiation (*PRKCZ*, *PDE6A*).

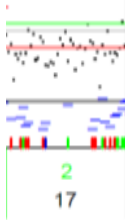
Table 1: Patients' characteristics.

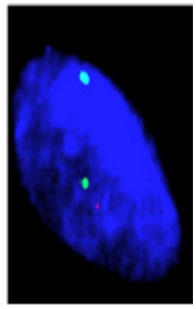
Characteristics	Total N (%)
Age	
50-59	14 (17)
60-69	27 (33)
70-79	24 (30)
80-89	16 (20)
Gender	
Male	34 (41)
Female	45 (54)
Tumor Location	
Right	52 (63)
Left	31 (37)
Stage	
I and II	54 (65)
III	29 (35)
MMR status	
MSS	55 (67)
MSI-H	27 (33)
Chr.17p allelic state	
AB	39 (47)
LOH	9 (11)
CN LOH	11 (13)
Amp LOH	7 (8)
AI	3 (4)
Multiple clones	14 (17)
DNA index	
0.95 – 1.05	35 (46)
1.06 – 1.40	10 (13)
1.41 – 1.95	31 (41)
TP53	
wt	22 (55)
mut	18 (45)
IHC p53	
0 %	10 (13)
>0% - ≤25%	35 (46)
>25%	31 (41)

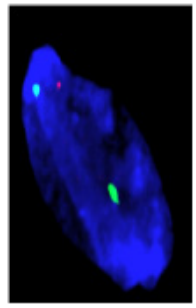
Sample 1: DNA index=1.1

a) b)





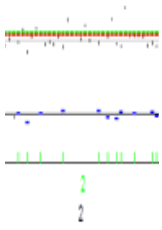


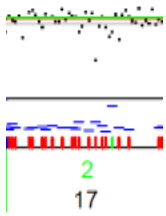


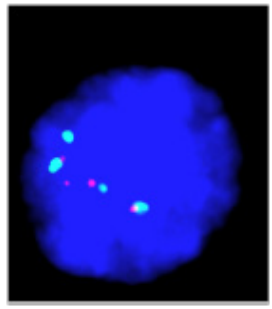
LAIR chr 2: AB LAIR chr.17: A FISH: two centromeres and one *TP53* copy

Sample 2: DNA index=2.3

a) b)







LAIR chr. 2: AABB LAIR chr.17 AAAA FISH: four centromeres and four *TP53* copies

Figure 2: Results of a) SNP array on reference chromosome and chr.17p
b) FISH on Chr. 17 (the green signal corresponds to the centromere probe and the red signal to the *TP53* probe).

Table 2: Associations between clinicopathological variables and p53 functionality.

	p53 non functional N (%)	p53 functional N (%)	p value
***TP53* mutational status**			
wt	7 (33)	14 (78)	0.01
mut	14 (67)	4 (22)	
P53 IHC			
0	3 (11)	7 (24)	<0.001#
0 - ≤25%	1 (3)	22 (76)	
>25%	24 (86)	0 (0)	
Chr. 17 p status			
AB	5 (18)	22 (76)	<0.001*
LOH	2 (7)	4 (14)	
Copy neutral LOH	9 (32)	0 (0)	
Amplified LOH	5 (18)	1 (3)	
Allelic Imbalance	1(4)	0 (0)	
Two clones	6 (21)	2 (7)	
Age category			
50 – 59	4 (14)	6 (22)	NS
60 – 69	10 (36)	9 (32)	
70 – 79	10 (36)	9 (32)	
80 – 89	4 (14)	4 (14)	
DNA index			
0.95 – 1.05	6 (22)	21 (78)	<0.001¶
1.06 – 1.4	4 (15)	3 (11)	
1.41 – 1.95	17 (63)	3 (11)	
MMR status			
MSI	4 (14)	14 (50)	0.009
MSS	24 (86)	14 (50)	
Gender			
Male	12 (43)	18 (62)	NS
Female	16(57)	11 (38)	
Tumor Location			
Right	10 (36)	19 (66)	0.035
Left	18 (64)	10 (34)	
Stage			
I and II	14 (50)	22 (76)	0.06
III	14 (50)	7 (24)	

*X^2 test allelic status AB vs. LOH p=0.58; **AB vs. CN LOH p<0.001**; **AB vs. Amp LOH p=0.005**; **AB vs. two clones p=0.006; LOH vs. CN LOH p=0.01**; LOH vs. Amp LOH p=0.24; LOH vs. two clones p=0.28; Amp LOH vs. CN LOH p=0.43; Amp LOH vs. two clones p=1; CN LOH vs. two clones p=0.48

X^2 test p53 IHC 0 vs. 0-25% p=0.07; **0 vs. >25% p<0.001; 0-25% vs. >25% p=0.001**

¶ X^2 test DNA index 0.95 – 1.05 vs. 1.06 – 1.4 p=0.16; **0.95 – 1.05 vs. 1.41- 1.95 p<0.001;** 1.06 – 1.40 vs. 1.41 – 1.95 p=0.29

Table 3: List of genes differentially expressed between functional p53 and non functional p53 groups.

Gene name	Chr. position	Gene description	p-value p53 functional vs. p53 non functional
PRKCZ	1p36.33-p36.2	Serine threonine kinase involved in several processes such as proliferation, differentiation and secretion.	4.95E-04 ↑non functional
LMO3	12p12.3	Lim domain only 3 (rhombotin like 2). Expression of LMO-3 represses p53 mediated mRNA expression of target genes.	1.2E-02 ↑non functional
CDKN1A	6p21.2	Cyclin dependent kinase inhibitor. Causes cell cycle arrest in the presence of DNA damage.	1.3E-02 ↑functional
PDE6A	5q31.2-q34	Phosphodiesterase 6A, cGMP-specific, rod, alpha	7.47E-02 ↑non functional
SIAH1	16q12	Seven in absentia homolog 1. Involved in ubiquitination and proteosome related degradation of specific proteins like beta catenin.	2.60E-02 ↑non functional
TPD52L2	20q13.2-q13.3	Tumor protein D52 like 2. Expressed in childhood leukemia and testes.	4.65E-02 ↑non functional
MDM2	12q14.3-q15	MDM2 p53 binding protein homolog (Mouse)	1.25E-02 ↑ functional
HMGCS1	5p14-p13	3-hydroxy 3-methylglutaryl-CoA synthase I	1E-01 ↑functional

All p-values are corrected for multiple testing.

Patient survival analysis

In a univariate survival analysis, p53 functionality was prognostic, patients with functional p53 had a better cancer specific survival than patients with non functional p53 (Log rank p=0.009) (figure 3).

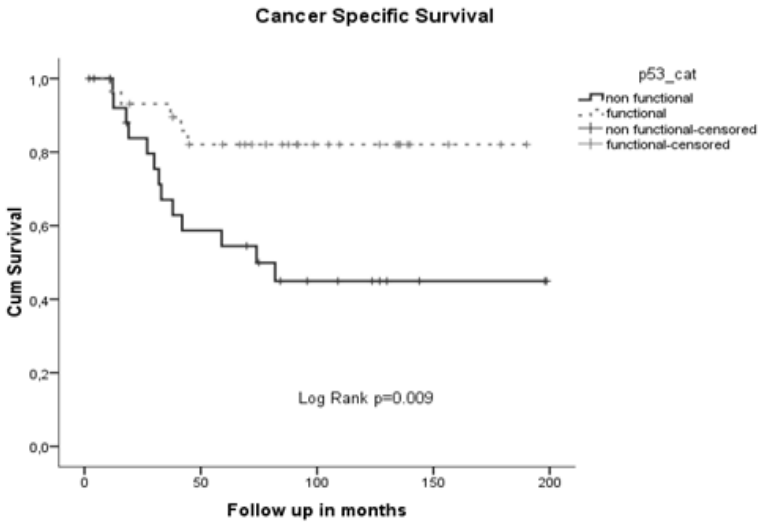
In the present cohort of patients, MSI-H was somewhat more frequent than expected from epidemiological studies (33% vs. 18% expected), nevertheless MMR status did not influence survival (data not shown) nor the effects of p53 functionality on survival. Recently, the role of p53 and Csnk1a1 inactivation in tumor invasiveness in mice has been demonstrated [3]. We analyzed whether the expression levels of *CSNK1A1* influenced p53 effects in disease outcome. For each of the three probes analyzed (A_23_P213551; A_24_P183292; A_24_P251899) patients were divided according to the expression level in high expression when expression level was greater than the median value for that specific probe and low expression when the value was lower than the median. The values of the three probes correlated significantly with each other (Pearson's correlation coefficient =0.94 p<0.001 between A_23_P213551 and A_24_P251899, 0.747 p<0.001 between A_23_P213551 and A_24_P183292 and finally 0.743 p<0.001 between A_24_P183292 and A_24_P251899) (figure 4). The three probes had the same detrimental effect on survival in a univariate analysis with different significant p values (data not shown). We selected the probe (A_24_P183292) with the most significant results (Log rank p=0.003) for further analyses.

CSNK1A1 expression significantly altered the effect of p53 in survival as shown in figure 5. *CSNK1A1* had no influence on survival when p53 is functional, however, if patients had a non functional p53, *CSNK1A1* expression influenced disease outcome dramatically. Patients with low *CSNK1A1* expression had a very poor prognosis compared with patients with high *CSNK1A1* expression (Log rank p=0.007) (figure 5).

We then classified patients in two categories based on p53 functionality and *CSNK1A1* expression; i.e. patients with non functional p53 and low *CSNK1A1* expression and the rest of patients (non functional p53 and high *CSNK1A1* expression or functional p53 with high or low *CSNK1A1* expression). Patients with both genes affected died earlier than patients with one of both genes active (figure 6) (Log rank p<0.001). Moreover, this detrimental effect on disease outcome was significant in a multivariate model including tumor stage, gender, tumor location and MMR status in the model (HR=4.74 95%CI 1.47-15.34 p=0.009) (Table 4).

Expression of invasiveness genes

Next we analyzed expression of eight genes reported by Elyada *et al* as upregulated in the double (p53 and Csnk1a1) knockout mice and involved in murine tumor invasiveness[3]. Two human genes, mainly *PLAT* (plasminogen activator tissue) and *PNLPRP1* (pancreatic lipase related protein 1) were significantly differently expressed between two groups of patients; the group with low *CSKN1A1* expression and non functional p53 vs the remaining group (with functional p53 and high or low *CSKN1A1* expression and non functional p53 and high *CSNK1A1* expression). *PLAT* was upregulated in the latter group (p=0.009) whereas *PNLPRP1* was higher expressed in the non functional p53 and low *CSNK1A1* expression (p=0.009).



Log Rank p53 functional vs. non functional p=0.009

p53 functional N=29; p53 non functional N=28

Figure 3: Kaplan Meier plots for CSS according to p53 functionality.

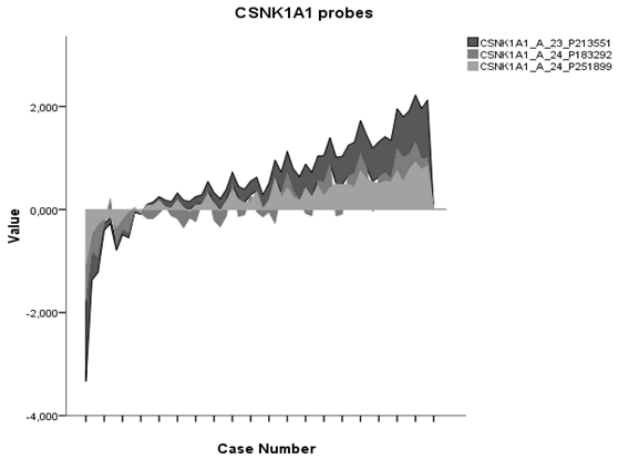
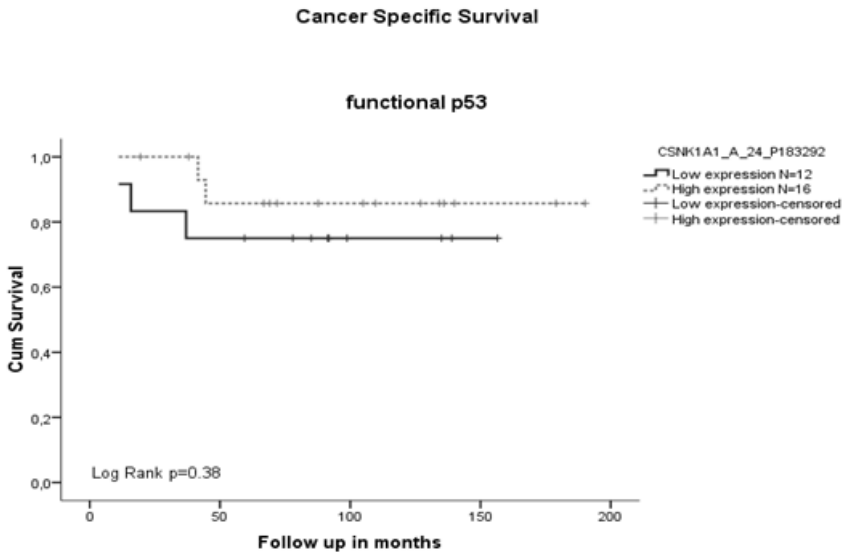
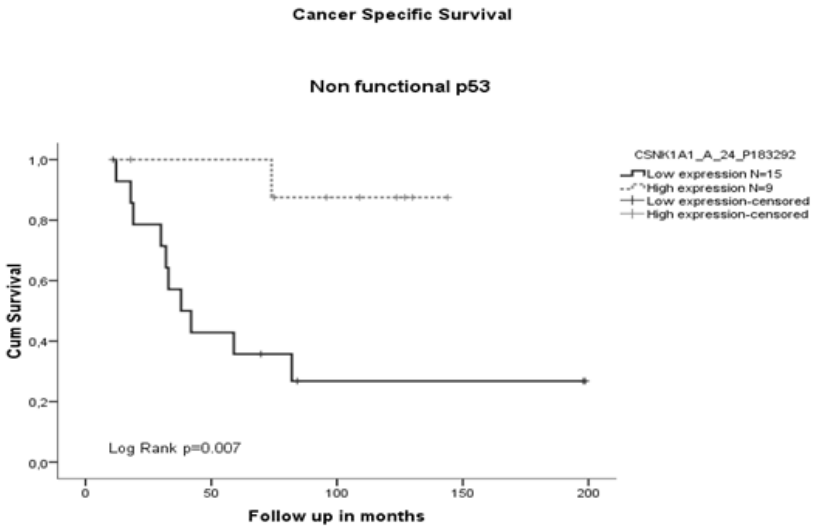


Figure 4: Trends in expression of the three *CSNK1A1* probes.



Log Rank high vs. low *CSKN1A1* expression p=0.38

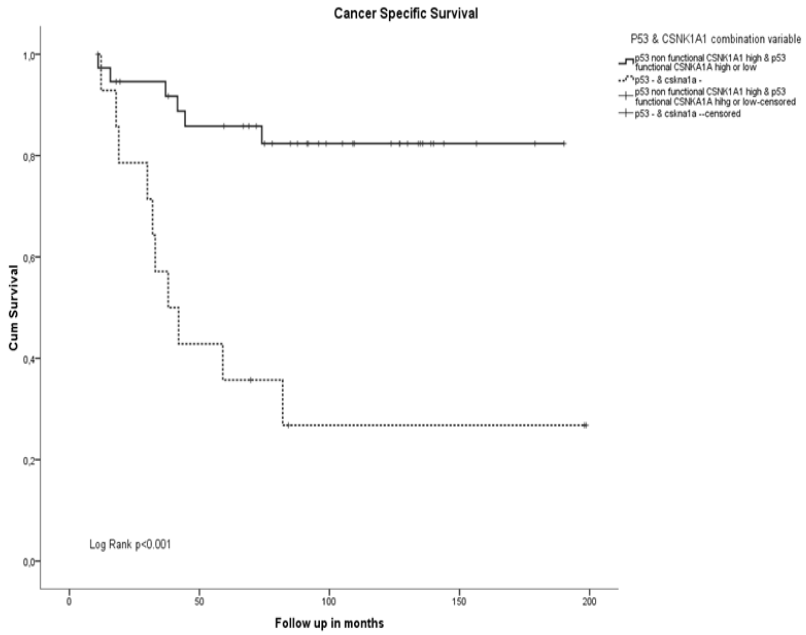
Low *CSNK1A1* expression N=12; High expression N=16



Log Rank high vs. low *CSNK1A1* expression p=0.007

Low *CSNK1A1* expression N=15; High expression N=9

Figure 5: Kaplan Meier plots for CSS according to *CSNK1A1* expression stratified on the base of p53 functionality.



p53+ & *CSNK1A1*+/- and p53- & CSNK1A1 + N=37

p53- *CSNK1A1*- N=15

Figure 6: Kaplan Meier for CSS according to p53 and *CSNK1A1* combination variable.

Table 4: Cox Proportional Hazards Model: multivariate survival analysis.

Variables	HR	95% CI	p value
p53 & CSNK1A1 status			
p53 - & *CSNK1A1* + and p53+ & *CSNKA1A* +/-	Referent		
p53 - *CSNK1A1* -	4.74	1.47 – 15.34	0.009*
Tumor stage			
I & II	Referent		
III	3.48	1.08 – 11.2	0.037*
Tumor location			
Right	Referent		
Left	0.92	0.32 – 2.67	0.58
Gender			
Male	0.92	0.32 – 2.97	0.88
Female	Referent		
MMR state			
MSS	0.43	0.097 – 1.91	0.27
MSI	Referent		

* Statistically significant results

Discussion

p53 is a transcription factor with important functions in cellular apoptosis, senescence, DNA damage repair, autophagy, aging and glycolysis [31-33]. Therefore, it is a strategic target for inactivation in cancer cells and indeed it is found mutated in approximately 50% of all tumors [4]. However, the consequences of p53 inactivation in disease outcome in colon cancer remain controversial and a matter of discussion. Differences in the techniques used to assess p53 alterations (IHC or mutation analysis), together with the many possible ways of p53 inactivation (deletion and dominant negative, loss or gain of function mutations) play a part in the inconclusive results. We studied *TP53* using several approaches; first we determined tumor ploidy and *TP53* locus allelic state. Next, we assessed *TP53* mutation state and protein expression by IHC. Integrating all these data we were able to reliably predict p53 functionality. The classification in functional and non functional p53 was ratified by the significant differences in target gene expression between these two groups. Known p53 targets such as *CDKN1A* and *MDM2* were significantly higher expressed in the p53 functional group than in the non functional, corroborating p53 functional state. Thus, with this approach complete information over the gene was obtained allowing a more reliable classification than by mutation analysis or immunohistochemistry solely.

As it could be expected based on the functions of p53, tumors with a non functional p53 were highly aneuploid and had a poorer prognosis than patients with functional p53.

We have also shown that p53 can indeed behave as a haploinsufficient tumor suppressor gene in humans as already seen in mice models [20]. By the use of SNP array we access the allelic state of the p53 locus and by additionally assessing *TP53* mutation state we were able to determine *TP53* genotype accurately. In our cohort there were a few patients with LOH at the *TP53* locus but without mutations in exons 5, 6, 7 and 8 and without positive immunostaining. Moreover, these patients had an almost diploid genome and all had a good disease outcome as compared with other patients. This finding supports what has been seen in mice, where p53 +/- mice did develop tumors but show a milder phenotype than p53-/- mice [20].

Recently, Csnk1a1 or CKIα expression has been implicated in colon cancer invasiveness and cell transformation in mice gut [3]. *CSNK1A1* is a serine/threonine kinase that phosphorylates β-catenin to target it for destruction [34]. In a mouse model, ablation

of Csnk1a1 caused the accumulation of β-catenin in the cytoplasm and nucleus activating many Wnt target genes although no tumor formation was observed. Instead, senescence was induced in these cells pointing to a possible role in tumor inhibition of p53. Indeed, the authors found that inactivation of both Csnk1a1 and p53 rendered the cell malignant and rapidly invasive [3]. Likewise, in the present cohort of patients, we have identified *CSNK1A1* as a dramatic modifier of p53 effects on survival. High *CSNK1A1* expression partly counteracts the negative effects of a non functional p53. Accordingly, low *CSNK1A1* expression and non functional p53 was equal to a very poor prognosis with a median survival time of 3 years and a 5-year survival of only 35%, which is extremely poor for early stage disease. Furthermore, this negative effect on survival was independent of disease stage, gender, tumor location and mismatch repair state, as shown in the multivariate analysis.

The exact mechanism behind this poor survival is unknown; Elyada *et al* showed that expression of certain genes was upregulated in the double knockout mice (p53-/- and Csnk1a1-/-) as compared with the only Csnk1a1-/- mice. Some of these genes were involved in loss of enterocyte polarity, tissue remodeling and cell motility; all functions likely to be involved in tumor invasiveness [3]. In the present cohort of patients only two of the human homologues from the murine gene list proposed were differentially expressed, i.e. plasminogen activator tissue (*PLAT*) and pancreatic lipase related protein 1 (*PNLRP1*) in tumors with impaired p53 function and low expression of *CSNK1A1* versus the remaining tumors. The latter results might reflect differences between mouse and man. Moreover, the human comparison was not identical to the murine comparison by Elyada and co workers. Furthermore in contrast to the murine model, *PLAT* was upregulated in the group with at least one active gene (functional p53 with low or high *CSNK1A1* expression and non functional p53 with high *CSNK1A1* expression) and could therefore be associated with a better survival. In human, the increased expression of the plasminogen activator inhibitor was associated with the occurrence of distant metastasis in colon cancer [35], probably leading to decreased levels of *PLAT* which would corroborate our findings. To our knowledge, the role of *PNLRP1* in tumor invasiveness and progression is so far unknown.

In conclusion, the combination of several approaches gives additional and accurate information on p53 status showing a detrimental effect on survival when p53 function is impaired. Nevertheless, gene interplay remains very important in tumor biology as it is illustrated by the modifying role of *CSNK1A1* gene expression on the survival effects

of *TP53* in colon cancer. Loss of both genes confers an extremely poor prognosis to colon cancer patients.

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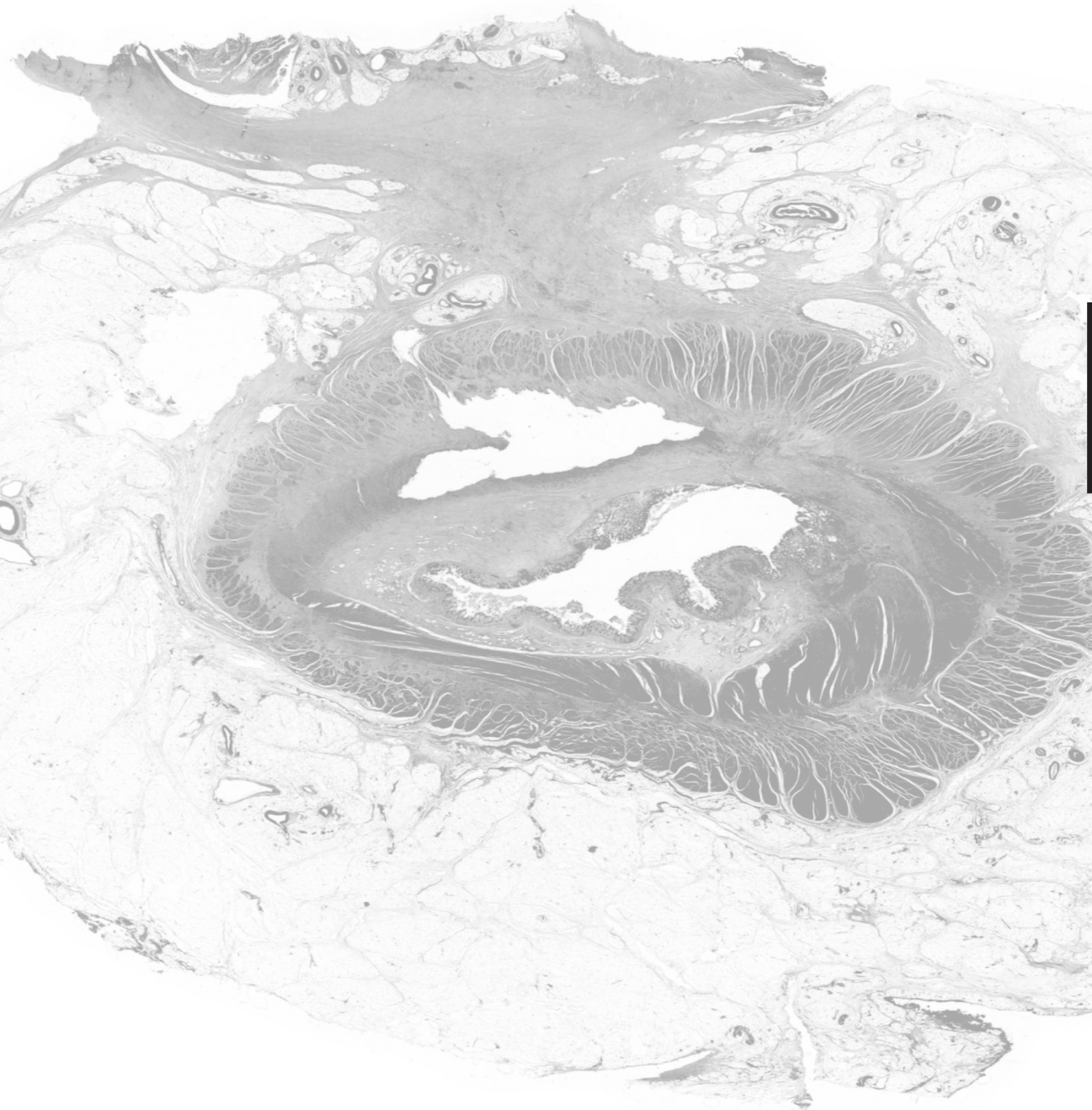

8

BRAF V600E mutated colon carcinoma associated genomic profile differs from double wild type tumors genome

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Abstract

Apart from its activating function in the MAPK transduction pathway, mutated BRAF has been recently implicated in mitochondrial oxidative phosphorylation, resistance to apoptosis, mitotic spindle alterations and aneuploidy. In colon cancer, V600E *BRAF* mutated tumors show different gene expression profile than *KRAS* and double wild type tumors. Moreover, the V600E *BRAF* mutation has also been associated with a poor prognosis.

We aimed to investigate the genomic profile of *BRAF* V600E mutated microsatellite stable (MSS) colon cancer tumors and compare it with double wild type MSS tumors. For this purpose, thirty four patients with stage II and III colon cancer were selected for tumor cell flow sorting, DNA isolation of normal and tumor fractions and hybridization to the high density Affymetrix Oncoscan™ FFPE Express SNP array.

BRAF mutated tumors have a different genomic profile than double wild type tumors. Concretely, the *BRAF* mutated tumors show more frequently gain of chromosome 18p (p=0.03) and 20q (p=0.03) and losses of chromosomes 3p (p=0.03), 6p (p=0.03) and 6q (p=0.02).

Introduction

BRAF is a serine threonine kinase involved in the KRAS/ERK transduction pathway. Several mutations in the *BRAF* gene have been described; however the mutation causing the substitution of valine by glutamic acid at position 600 of the protein is the most frequent one, representing 95% of the *BRAF* mutations. In colon cancer the *BRAF* c.1799T>A, p.V600E mutation does not coexist with *KRAS* mutations. This observation together with the fact that these two proteins function in the same signaling pathway suggest possible overlapping functions of KRAS and BRAF. However, compared to *KRAS* mutated or double wild type tumors, the *BRAF* V600E mutated tumors show a different phenotype consisting in poor differentiation, microsatellite instability and proximal location in the colon. Moreover, the pattern of metastatic spread of *BRAF* mutated tumors seems to be different than that of non *BRAF* mutated tumors. Tumors with a *BRAF* mutation spread more frequently to the peritoneum, distant lymph nodes and less frequently to the lung regardless of the microsatellite status. Liver and central nervous system metastases rates did not differ between *BRAF* mutated and wild type[1]. The *BRAF* V600E mutation has been associated with poor prognosis in early stages of colon cancer, mainly in microsatellite stable (MSS) tumors [2-4] and with prognosis and no response to anti-EGFR therapy in metastatic colon cancer [5,6]. Recently, a specific *BRAF* V600E gene expression signature in colon cancer has been described [7]. This profile based on the expression of approximately 30 genes seemed to differ greatly from the *KRAS* mutation profile based on the expression of 90 genes and also from double wild type tumors. Based on this evidence, one could state that *BRAF* mutated tumors are indeed different entities as compared with *KRAS* mutated and double wild type tumors. Furthermore, oncogenic *BRAF* might have more functions than protein phosphorylation in signal transduction: the BRAF V600E mutant protein has also been implicated in dysregulation of apoptosis in colon cancer [8] and recently with mitotic spindle aberrations leading to chromosomal instability in melanoma cell lines. The transfection of melanoma cell lines with a construct containing *BRAF* V600E caused aberrant mitotic spindles, altered centrosomes and missegregation of chromosomes leading to aneuploidy. These mitotic spindle alterations were reversed by *BRAF* V600E inhibition [9]. Moreover, in thyroid cancer *BRAF* V600E has been found to locate at the mitochondria, suggesting a possible role in apoptosis and oxidative phosphorylation [10].

We hypothesized that *BRAF* mutant colon cancers could have other genomic aberrations

than double wild type tumors and that these genomic aberrations could be responsible for the poor prognosis of these patients. In order to obtain the most reliable results, we purified the epithelial tumor fraction of double wild type and *BRAF* V600E mutated samples by flow cytometry, isolated DNA and performed a 300K SNP array. Our results show indeed different genomic signatures for *BRAF* mutant tumors as compared with double wild type tumors.

Patients and methods

Patients

Colon carcinoma tissue of 34 patients diagnosed at the PAMM laboratory for pathology in Eindhoven, the Netherlands were selected on the basis of microsatellite stability (MSS), *BRAF* V600E mutation status, and being wild type for *KRAS* at codons 12 and 13 [2]. Six patients had stage II disease whereas 28 had stage III. Twenty of the stage III patients (71%) had received adjuvant chemotherapy previously to tissue collection (two received 5-FU combined with oxaliplatin, whereas the rest received 5-FU/LV). All of the stage II patients and eight of the stage III were treated with surgery solely.

Half of the patients where double wild type for both *BRAF* and *KRAS,* while the other half had a BRAF V600E mutation. Variables like age, gender, T and N stage, tumor differentiation etc. were matched between the two groups. However, it was not possible to match tumor location because the presence of a *BRAF* V600E mutation correlates with tumor location on the right side of the colon. Also, the selected *BRAF* mutated samples had a worse disease outcome than no *BRAF* mutated samples. Follow up data were obtained from the available medical records.

Tissue preparation for multiparameter flow cytometry and sorting

Tissue preparation for flow cytometry was carried out as previously described with minor modifications [11]. Tumor areas were selected by an experienced pathologist (IvL). Subsequently, 2 mm punches were taken and embedded in new blanco paraffin acceptor blocks. 60µM sections were then sliced, deparaffinised and rehydrated followed by heat induced antigen retrieval which was performed by warming the tissues for one hour at 80°C in 10mM sodium citrate (pH=6.0). Next, tissues were dissociated enzymatically with a mixture of collagenase and dispase and mechanically with the gentleMACS™ mechanical dissociator (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) until a cell suspension was obtained. Cells were then counted and primary antibodies added; mouse monoclonal AE1/AE3 (Millipore, Temecula, CA, USA) and MNF116 (DAKO, Golstrup, Denmark) pankeratin and mouse monoclonal V9-2b against vimentin (antibodies for research applications, Gouda, the Netherlands) in 1:200, 1:100 and 1:50 dilutions respectively. After overnight incubation, secondary antibodies were added, GaMIgG1-FITC and GaMIgG2b-APC both diluted 1:100 (Southern Biotech, Birmingham, Al, USA) followed by the DNA staining solution containing 10 µM propidium iodide

and 0,1% RNAse (Sigma-Aldrich, Zwijndrecht, the Netherlands). Cells were then sorted using the FACS ARIA I and the FACS ARIA SORP (BD Biosciences, San Jose, CA, USA) based on cellular vimentin, keratin expression and DNA content.

DNA index (DI) defined as the ratio between the median G_0/G_1 keratin epithelial fraction and the median G_0/G_1 vimentin stromal fraction, was calculated using a remote link between Winlist 6.0 and ModFit 3.21. (Verity Software House) for each sample. Whenever, more than one keratin positive population was seen, it was independently sorted. DI was categorized as DI< 0.95 or DNA hypodiploid; DI=0.95 – 1.05 DNA near diploid; DI=1.06 – 1.4 DNA aneuploid and DI=1.41 – >1.95 DNA tri or tetraploid.

Harvested cells were centrifuged for 20 minutes at 13.000 rpm and kept at -80°C until DNA isolation. DNA was isolated after proteinase K digestion at 56°C overnight and purified with Nucleospin Tissue (Marcherey Nagel, Düren, Germany) following manufacturer's instructions. DNA concentration was adjusted to 15 ng/µL according to picogreen® measurements.

High density SNP array OncoScan™ FFPE Express

Two samples (one tumor and one normal sample) were excluded from further analysis because of their poor DNA quality. Sixty-six samples (33 normal and 33 tumor samples) as well as their matched normal DNA were hybridized on the OncoScan™ FFPE Express (Affymetrix, Inc. Santa Clara, CA, USA). This array challenges 330000 SNP markers in the genome with an average markers spacing of 9119 bp. After the hybridization, sixteen double wild type samples and fifteen *BRAF* V600E mutated samples were available for analysis.

Statistical Analysis

In order to identify genomic regions with identical copy number, normalized allele intensities (as provided by Affymetrix) were segmented using the circular binary segmentation[12].

After all samples have been segmented the overlapping segments across all samples were reduced to unique segments. For both whole genome and chromosomal arms, we used the global test to evaluate for presence of differences in copy number between *BRAF* mutated and double wild type[13] . Differences between groups were accepted as significant with a false discovery rate lower than 0.05.

Association between categorical variables was calculated by the χ^2 Fischer Exact Test with SPSS v16 for Windows (SPSS, Inc. Chicago, Il. USA).

Results

The clinical and epidemiological characteristics of the thirty-four patients are shown in table 1. As previously stated, there were no differences in the distribution of the clinical and epidemiological variables between the two groups except for tumor location and disease outcome.

DNA index was determined for each sample during FACS. Six samples (18%) had two keratin positive populations with different DNA indexes. The distribution of the different DNA indexes according to the mutation status is shown in figure 1. Globally, the median DNA index was 1.54 (0.94 – 1.97). DI categories were made as follows, 9% DNA hypodiploid (DI<0.95); 12% DNA near diploid (≥0.95 – 1.05); 18% DNA aneuploid (1.06 – 1.40); 62% DNA triploid/tetraploid (1.41 - >1.95). There were no significant associations between number of cell populations and DI with *BRAF* mutational status or with other clinical or epidemiological variables.

After hybridization on the array, data was available from sixteen double wild type samples and fifteen *BRAF* mutated samples which represent a success rate of 90%.

Previously described genomic aberrations in colon cancer are seen in both groups without significant differences[14-20] (figure 2, table 2).

On average *BRAF* mutated samples showed a higher number of genomic fragments than double wild type samples, although no statistical significance was reached possible due to the low sample size. Nevertheless, the copy number profile is significantly different between *BRAF* V600E mutated and double wild type samples (p=0.002). In order to investigate which chromosomal arm contribute to the overall difference between the two groups of patients, it was observed that alterations in chromosomes 3p, 6p and 6q, 18p and 20q were significantly different between both groups (see table 3). When compared with the double wild type group, chromosome losses were more frequently in the *BRAF* mutated group in 3p, 6p, and 6q (p=0.03, p=0.03 and p=0.02), while chromosome gain were more frequent in 18p and 20q (p=0.03 for both alterations).

Table 1: Patient's clinicopathological characteristics.

Variables	Total N (%)	Double wt N (%)	*BRAF* V600E N (%)
BRAF			
wt	17 (50)	17 (100)	0 (0)
V600E	17 (50)	0 (0)	17 (100)
PIK3CA			
wt	29 (85)	14 (82)	15 (88)
p.E545K	5 (15)	3 (18)	2 (12)
KRAS			
wt	34 (100)	17 (100)	17 (100)
mut	(0)	0 (0)	0 (0)
T status			
T2	1 (3)	1 (6)	0 (0)
T3	27 (79)	14 (82)	13 (76.5)
T4	6 (18)	2 (12)	4 (23.5)
Nodal status			
N0	6 (18)	3 (18)	3 (18)
N+	28 (82)	14 (82)	14 (82)
Differentiation category			
Well/Moderately differentiated	26 (76.5)	12 (71)	14 (82)
Poorly/undifferentiated	8 (23.5)	5 (29)	3 (18)
Gender			
Male	18 (53)	10 (59)	8 (47)
Female	16 (47)	7 (41)	9 (53)
Tumor location			
Right	21 (62)	8 (47)	13 (76.5)
Left	13 (38)	9 (53)	4 (23.5)
Follow up status *			
No Evidence of Disease	18 (53)	13 (76)	5 (29)
Alive With Disease	1 (3)	1 (6)	0 (0)
Dead of Disease	12 (35)	3 (18)	9 (53)
Dead of Other Cause	3 (9)	0 (0)	3 (18)
Median age (range)	66 (44 – 79)	64 (44 - 79)	69 (46 - 77)
Median Follow up (range)	44 (0 – 96)	61 (22 – 96)	35 (0 – 93)

* Statistically significant p=0.017

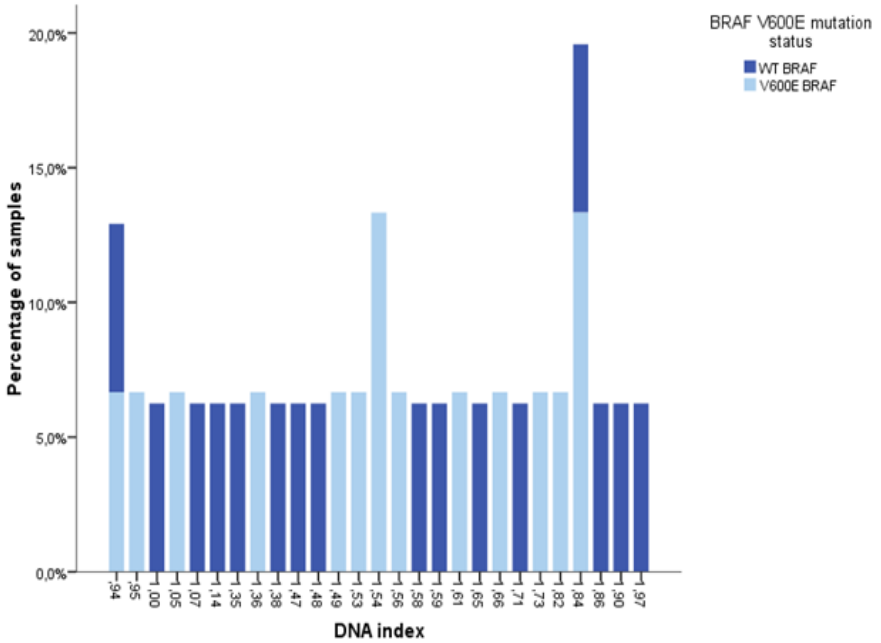

Figure 1: DNA index according to *BRAF* mutation status.

Table 2: Known genomic alterations in colon cancer. Differences between *BRAF* mutated group and double wild type group.

Chromosome arms	p-value
CN chr. 1p loss	0.65
CN chr.1q loss	0.9
CN chr. 8p loss	0.8
CN chr. 8q gain	0.52
CN chr.13 gain	0.13
CN chr. 17p loss	0.73
CN chr.18q loss	0.17
CN chr.22 loss	0.27

Table 3: Specific genomic regions differently affected between double wild type and *BRAF* V600E mutated samples.

Chromosome arms	p-value	Corrected p-value
Chr. 3p	0.00259	0.025
Chr.6p	0.00139	0.02
Chr.6q	0.0003	0.01
Chr.18q	0.0018	0.0252
Chr.20p	0.0028	0.0252

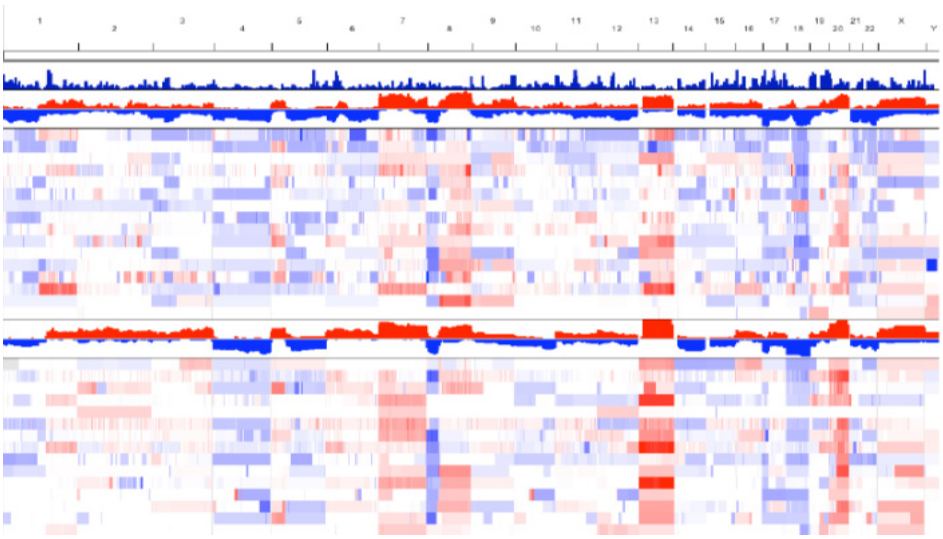

Figure 2: Genomic profile *BRAF* V600E mutated group (upper panel) vs. double wild type group (lower panel). Upper row represents chromosome numbers; second row gene density according to the RefSeq database; third row, the mean gains and losses for the *BRAF* V600E group, as the row between panels for the double wild type group. Genomic gains are indicated in red, losses in blue, with a summarizing scheme above each pannel. The genomic profile of the *BRAF* V600E mutated tumors group differs significantly from that of the double wild type tumors.

Discussion

Based on our results we can conclude that *BRAF* mutated colon cancer tumors have a distinctive genomic profile as compared with double wild type colon cancer tumors. Focal regions on chromosome 18p, 20q, 3p, 6p and 6q were differently affected in the *BRAF* mutated samples as compared with the double wild type samples. Furthermore, *BRAF* mutated tumors seemed to be more aberrant than double wild type tumors as the number of genomic fragments causing a so called "scattered genomic pattern" was greater than in double wild type specimens albeit not yet statistically significant.

The scattered pattern could be due to the induction by oncogenic *BRAF* of pleiotropic spindle abnormalities leading to chromosome missegregation and aneuploidy as described by Cui *et al*[9]. As stated by the authors, the abnormalities caused by oncogenic *BRAF* are expected to occur at random explaining the scattered pattern found in this study. At the same time, this mechanism could not be responsible for the specific chromosomal alterations in chr.18p, chr.20q, chr.3p, chr.6p and chr.6q seen in the *BRAF* mutated group; raising the question of what could be the mechanism behind these alterations. Does *BRAF* play a causative role on it, facilitating selection of mutated clones and hence a more aggressive disease history or does it just constitute together with the other alterations a signature identifying a type of colon cancer with a very aggressive course?

Loss of 6q has been independently associated with the development of colon cancer and with a more aggressive form of the disease. To our knowledge there is no direct association described between chr.3p loss and colon cancer; however, many genes are affected by the loss of this region like for instance *FOXP1*. Overexpression of *FOXP1* has been associated with a better disease outcome in breast cancer patients[21]. Possibly, loss of this gene as seen in the *BRAF* V600E mutant group might explain a poorer prognosis, although this last point remains to be proven.

To our knowledge there are no reports over the other alterations and prognosis or *BRAF*.

In conclusion, in this study we show for the first time that *BRAF* V600E mutated and double wild type colon tumors show subtle differences at the genomic level. In general, *BRAF* mutated tumors have a different genomic profile than double wild type tumors. Moreover, these tumors show different focal and regional alterations, with gain of chromosome 18p, 20q and losses of chromosomes 3p, 6p and 6q affecting

genes involved in cancer formation or progression which are probably not caused by *BRAF* V600E itself. Whether these specific focal aberrations are caused by *BRAF* V600E directly or whether they constitute together with *BRAF* V600E itself a specific genomic profile of highly aggressive tumors remains unanswered and subject of future research.

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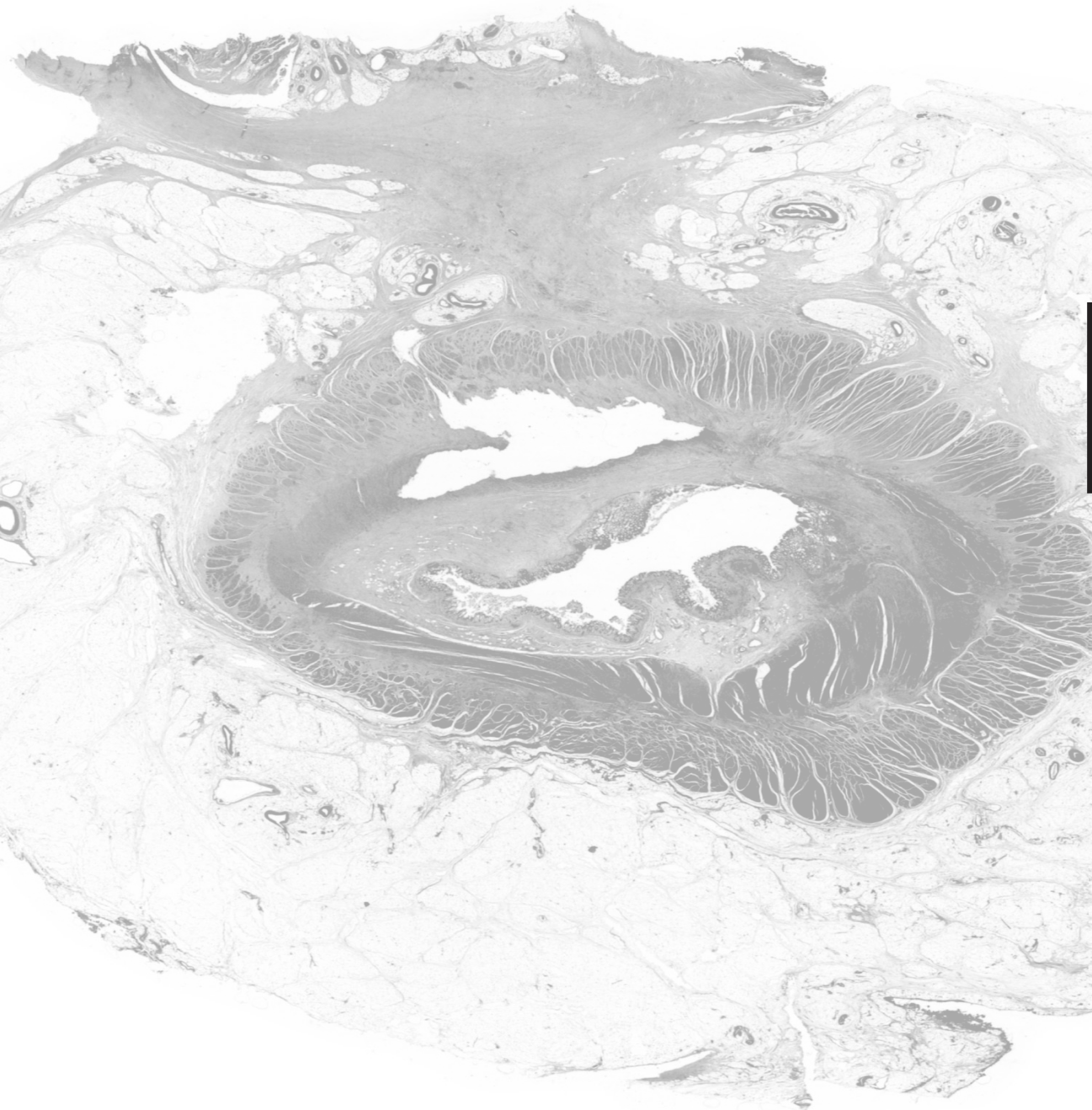

9

SNaP shot and StripAssay™ as valuable alternatives to direct sequencing for *KRAS* mutation detection in colon cancer routine diagnostics

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Abstract

Although, direct sequencing is the gold standard for *KRAS* mutation detection in routine diagnostics, it remains laborious, time consuming and not very sensitive.

Our objective was to evaluate SNaPshot and the *KRAS* StripAssay™ as alternatives to sequencing for *KRAS* mutation detection in daily practice.

KRAS exon 2 specific PCR followed by sequencing or by a SNaPshot reaction was performed. For the StripAssay™, a mutant enriched PCR was followed by hybridization to *KRAS* specific probes bound to a nitrocellulose strip. To test sensitivities, dilution series of mutated DNA in wild type DNA were made. Additionally, direct sequencing and SNaPshot were evaluated in 296 colon cancer samples.

Detection limits of direct sequencing, SNaPshot and StripAssay™ were 20%, 10% and 1% tumor cells, respectively. Direct sequencing and SNaPshot can detect all 12 mutations in *KRAS* codons 12 and 13, whereas the StripAssay™ detects ten of the most frequent ones. Workload and time to results are comparable for SNaPshot and direct sequencing. SNaPshot is flexible and easy to multiplex. The StripAssay ™ is less time consuming for daily laboratory practice.

SNaPshot is more flexible and slightly more sensitive than direct sequencing. The clinical evaluation showed comparable performances between direct sequencing and SNaPshot. The StripAssay™ is rapid and an extremely sensitive assay which could be considered when few tumor cells are available. However, found mutants should be confirmed to avoid risk of false positives.

Introduction

Since the introduction of targeted therapy against the epidermal growth factor receptor (EGFR) for the treatment of metastatic colorectal cancer, mutation detection in downstream effector molecules like *KRAS* has become clearly more important in clinical practice. It has been well reported in literature that patients harbouring mutations in these molecules will not benefit from anti-EGFR treatment[1, 2]. Several mutations have been described in the *KRAS* gene, impairing response to anti-EGFR therapy. These mutations occur most frequently (97%) in codons 12 and 13 of exon 2 (the first coding exon); less common (3%) are the mutations in codons 59 and 61 in exon 3[3]. The clinical value of these latter mutations is still unknown. *KRAS* mutations occur early in colorectal carcinogenesis and are present in 30 up to 40% of colorectal carcinoma cases, independently of disease stage[4].

Recently, the American Society of Clinical Oncology (ASCO) has issued the recommendation to test for *KRAS* mutations in all patients with metastatic colorectal cancer before treatment with cetuximab[5]. Moreover, in Europe *KRAS* mutation analysis in stage II and III colon cancer has been recommended by an expert panel[6]. Thus, *KRAS* mutation detection plays an important role in colon cancer therapy decision making and could very well become one of the most frequently performed tests in diagnostic pathology laboratories in the future.

Accurate mutation detection depends on several factors, including available tissue, DNA quality, DNA input and tumor cell percentage. All are important issues in limiting assay performance and sensitivity. The majority of assays in clinical practice are performed on formalin fixed paraffin embedded (FFPE) resection material. DNA from FFPE material is often of poor quality, impairing the performance of existing assays. Furthermore, DNA input can be a problem when little tissue is available as in needle biopsies. In addition, small numbers of tumor cells in a background of stromal cells can sometimes be challenging for accurate mutation detection as in the case of radio- and/or chemotherapy pre-treated tumor specimens.

When choosing an assay for routine diagnostics, additional factors such as workload, time to results, hands-on time, dedicated equipment, costs, assay flexibility and robustness of a technique need to be addressed as well. Assay flexibility enables multiplexing resulting in mutation detection on several hotspots or genes at the same time, saving diagnostic time and DNA input. Assay robustness or reproducibility is

mandatory to implement it in high throughput routine diagnostics. Finally, additional factors influencing technique choice are the capacity, equipment present and available expertise in a laboratory.

In most of the pathology laboratories direct sequencing, i.e. PCR followed by dideoxy sequencing, is considered as the gold standard for *KRAS* mutation detection. However, this technique is not only laborious and time consuming, sensitivity plays an important role. In order to reliably test a sample at least 20 to 30% of tumor cells are needed. To date, there are several alternative assays available for (*KRAS*) mutation detection, including home brew assays like high resolution melting curve analysis (HRM)[7], pyrosequencing[8], single nucleotide primer extension assay[9] allele specific real time PCR[10] and commercially available assays like reverse hybridization test *KRAS* StripAssay™ (Vienna labs, Vienna, Austria)[11] and real time PCR based TheraScreen™ (Roche Diagnostics, Almere, the Netherlands); all these assays greatly differ in sensitivity, specificity, DNA input, time to results, hands-on time, flexibility, workload and costs. The single nucleotide primer extension (SNaPshot) assay is a home brew, flexible assay, which might be easily extendable to other biomarkers, whereas from the commercially available assays the *KRAS* StripAssay™ claims to be fast and very sensitive.

Therefore in this study we aimed to evaluate the SNaPshot and reverse hybridization StripAssay™ in comparison to direct sequencing for *KRAS* mutation detection in colon cancer. Several parameters important for implementation in a pathology laboratory such as sensitivity, specificity, workload, time to results, hands-on time, flexibility, DNA input and costs have been compared.

Materials & Methods

Materials

In order to test the workload, time to results, hands-on time, costs, flexibility and specificity, 296 colon cancer samples available in the archives of the laboratory for pathology PAMM Eindhoven in the south of the Netherlands were used. Areas with sufficient tumor cell percentage were selected from diagnostic HE slides by an experienced pathologist. Percentages of tumor cells varied from 20 to 90%. These areas were macrodissected after tumor cell content check in new sandwich HE slides. Tissue input for DNA isolation was approximately 0.5 cm^2.

DNA was isolated by proteinase K digestion at 56°C overnight followed by purification with the HPTTP kit following manufacturer's instructions (Roche, Almere, the Netherlands).

To test the sensitivity of each assay, four different dilution series of mutant tumor DNA in wild type DNA were made. Five different mutations (c.34G>T; p.Gly12Cys, c.38G>A; p.Gly13Asp, c.35G>A; p.Gly12Val, c.35G>A; p.Gly12Asp and c.34G>C; p.Gly12Arg) were represented in these series. Tumor cell percentages of 80, 40, 20, 10, 5 and 1% were tested with the three assays.

To investigate possible false positivity of the StripAssay™, additional samples were tested. DNAs from eighteen samples containing a minimum of 75% tumor cells and previously diagnosed as wild type by direct sequencing and SNaPshot and two normal colonic mucosa samples were isolated following the same protocol as previously described. Subsequently, PNA PCR clamping was performed. The obtained PCR products were hybridized to the StripAssay™ strip and sequenced.

KRAS PCR and dideoxy sequencing

PCR for the amplification of codons 12 and 13 in exon 2 was performed using the primers described elsewhere[12]. The expected product length was 170 bp. Subsequently, 206 PCR products were purified using the QIAquick gel extraction kit (Qiagen, Venlo, the Netherlands) following manufacturer's instructions whereas 90 PCR products were purified by the enzymatic reaction with ExoSapIT (USB Co, Staufen, Germany). The change in purification method was due to the less laborious character of enzymatic purification, not affecting quality of sequence results. Purified products were then sequenced using the same primers as for the amplification and Big Dye

Terminator v1.1 cycle sequence kit (Applied Biosystems, Nieuwerkerk aan de IJssel, the Netherlands). Sequencing products were separated in the ABI 3100 and analyzed using the Sequencing Analysis 5.3.1 software (Applied Biosystems, Nieuwerkerk aan de IJssel, the Netherlands). Based on the fact that in our laboratory we have not found any discrepancy between *KRAS* mutation detection in wild type *KRAS* cases by sequencing with the forward or the reverse primer and to decrease workload, reactions were initially performed with the reverse primer. When a mutation was found, this was confirmed in a newly generated PCR product using the forward primer.

KRAS SNaPshot

PCR was performed using the same primer pair as for dideoxy sequencing[12]. Subsequently, products were purified with ExoSapIT (USB, Staufen, Germany). Next the single nucleotide primer extension reaction was performed as previously described [9] by adding four different oligonucleotides for each mutation hotspot and allowing the addition of a specific ddNTP differently labelled (figure 1). The following oligonucleotides were used 5'AAC TTG TGG TAG TTG GAG CT3' 5'N10ACT TGT GGT AGT TGG AGC TG 3' 5'N20TTG TGG TAG TTG GAG CTG GT 3' and 5'N30 TGT GGT AGT TGG AGC TGG TG3'. Primer extension reaction was performed according to manufacturer's instructions using the ABI PRISM SNaPshot™ multiplex kit (Applied Biosystems, Nieuwerkerk aan de IJssel, the Netherlands). Finally, products were run by capillary electrophoresis in an ABI 3100 and analyzed using the Genemapper v4.0 software (Applied Biosystems, Nieuwerkerk aan de IJssel, the Netherlands).

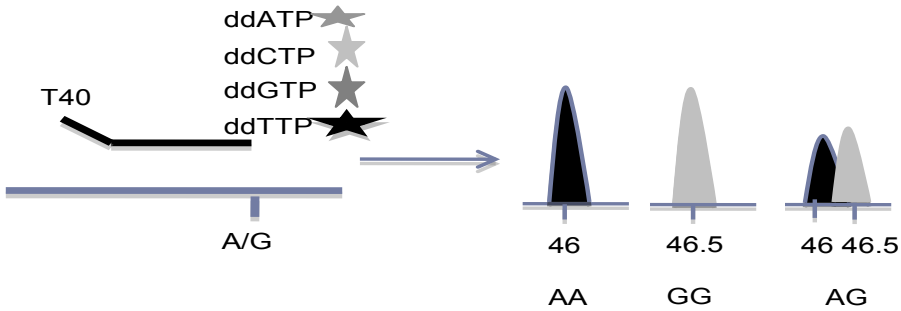

Figure 1: Schematic representation of the SNaP shot technique.

The primers have a certain length and end one nucleotide before the mutation. Subsequently, one fluorochrome labelled dideoxynucleotide is added. Using capillary electrophoresis products are separated according to size. Depending on the nucleotide build in after primer extension either one or two of the fluorochromes will be detected depending on the genotype.

KRAS StripAssay™

The *KRAS* StripAssay™ as recently described by Ausch *et al*[11] was performed according to manufacturer's instructions (Vienna Labs, Vienna, Austria). Briefly summarized, a PCR enriched for mutant *KRAS* alleles is performed. This PCR is based on wild type sequence clamping with a specific PNA oligonucleotide, allowing preferred amplification of the mutant sequence[13; 14]. Subsequently, PCR products are hybridized to a nitrocellulose strip containing specific probes for the different mutations (figure 2). After hybridization, the test strip is washed, blocked and color is developed[11].

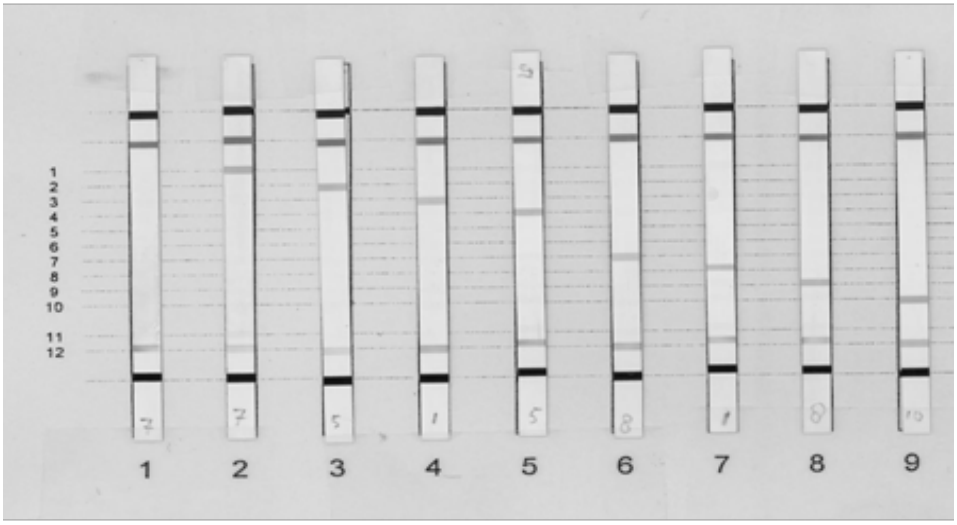

Figure 2: *KRAS* mutations present on StripAssay™.

1 Wild type; 2 p.Gly12Ala; 3 p.Gly12Arg; 4 p.Gly12Asp; 5 p.Gly12Cys; 6 p.Gly12Ser; 7 p.Gly12Val; 8 p.Gly13Asp; 9 p.Gly13Cys

p.Gly12Ile and p.Gly12Leu are not present in our series but present on the StripAssay™.

p.Gly13Val and p.Gly13Arg are not present on the StripAssay™.

Results

Technical validation: Sensitivity, specificity and performance.

Sensitivity

The sensitivity of three techniques, i.e. direct sequencing, SNaPshot and StripAssay™ was determined for *KRAS* mutation detection using different dilution series of mutated DNA with wild type DNA ranging from 80% to 10% or to 1% tumor cells. Different mutations, i.e. c.34G>C; p.Gly12Arg, c.34G>T; p.Gly12Cys, c.38G>A; p.Gly13Asp, c.35G>T; p.Gly12Val and c.35G>A; p.Gly12Asp were used for the dilution series.

A reproducible and reliable mutation detection limit of 20% tumor cell percentage was obtained for direct sequencing (see table 1 and figure 3). As shown in table 1, in two samples, mutation detection by direct sequencing was positive with only 10% tumor cells. However, reproducible results were not possible with less than 20%. The sensitivity of the SNaPshot assay was 10% tumor cells in the sample (see table 1, figure 3 and 4). Finally, the StripAssay™ appeared to be the most sensitive technique with a mutation detection limit of 1% tumor cells (table 1 and figure 4).

To investigate possible false positivity of the StripAssay™, additional samples, known to be wild type *KRAS* by direct sequencing and SNaPshot were tested by the StripAssay™ and sequencing of the clamped PCR product. Two conflicting results were found. Mutations were seen only by sequencing but products did not hybridize to the nitrocellulose strip. The mutations found were c.34G>A; p.Gly12Ser and c.39C>A with no aminoacid substitution. These samples were tested again and no mutants were found, neither with the StripAssay™ nor by direct sequencing.

Specificity

Previously tested samples with known mutations were used to check specificity of the different techniques. Although, c.37G>C; p.Gly13Arg, c.37G>A; p.Gly13Ser and c.38G>C; p.Gly13Ala were not seen in our samples, we believe that they are detectable with direct sequencing and SNaPshot just like the other nine mutations in codons 12 and 13 which were detected by both sequencing and SNaPshot. Of the mutations present in our series, the StripAssay™ failed to detect the c.38G>T; p.Gly13Val mutation because it is not present on the strip (Figure 2).

Table 1: Results of *KRAS* mutational analysis using Dideoxy sequencing, SNaPshot and StripAssay™ in five different tumor samples diluted with normal DNA.

Dilution series	Tumor percentage	Dideoxy Sequencing Forward primer	Dideoxy sequencing Reverse primer	SNaPshot	StripAssay™
c.34G>T	80	mut	mut	mut	mut
c12 GGT>TGT	40	mut	mut	mut	mut
p.Gly12Cys	20	mut	mut	mut	mut
	10	not detected	not detected	mut	mut
	5	not done	not done	mut	mut
	1	not done	not done	not detected	mut
c.38G>A	80	mut	mut	mut	mut
c13 GGC>GAC	40	mut	mut	mut	mut
p.Gly13Asp	20	mut	mut	mut	mut
	10	mut	mut	mut	mut
	5	not done	not done	not detected	mut
	1	not done	not done	not detected	mut
c.35G>T	80	mut	mut	mut	mut
c12 GGT>GTT	40	mut	mut	mut	mut
p.Gly12Val	20	mut	not detected	mut	mut
	10	not detected	not detected	mut	mut
	5	not done	not done	not detected	mut
	1	not done	not done	not detected	mut
c.35G>A	80	mut	mut	mut	Not done
c12 GGT>GAT	40	mut	mut	mut	Not done
p.Gly12Asp	20	mut	mut	mut	Not done
	10	mut	mut	mut	Not done
c.34G>C	80	mut	mut	mut	Not done
c12 GGT>CGT	40	mut	mut	mut	Not done
p.Gly12Arg	20	not detected	not detected	mut	Not done
	10	not detected	not detected	mut	Not done

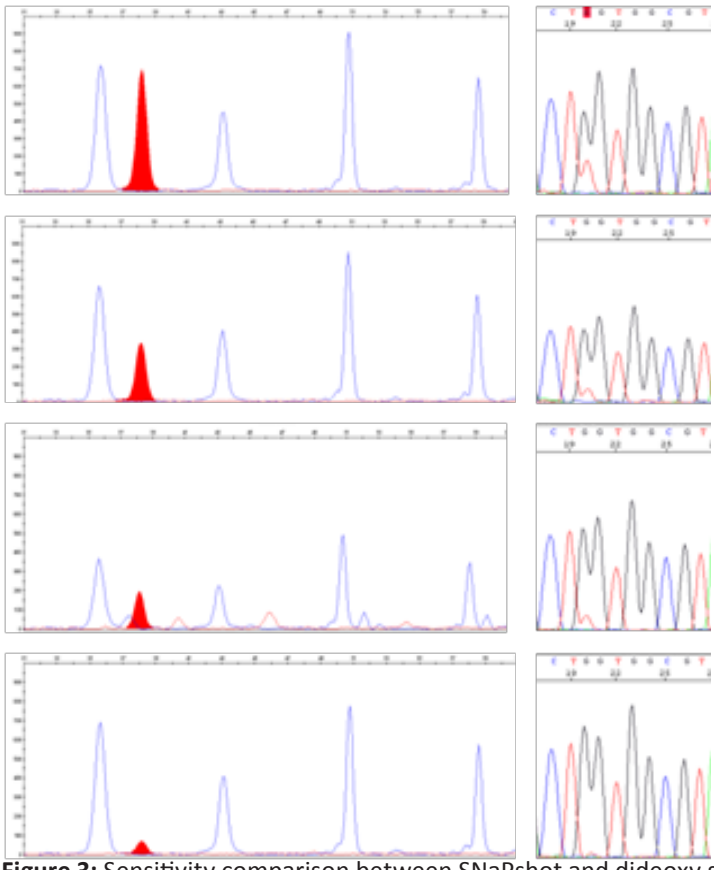

Figure 3: Sensitivity comparison between SNaPshot and dideoxy sequencing.

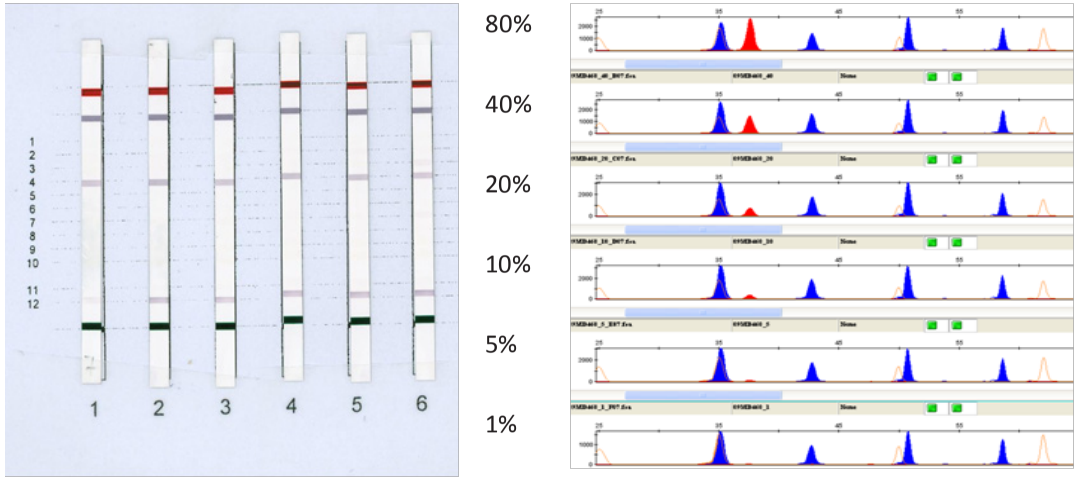

Figure 4: Sensitivity comparison between StripAssay™ and SNaPshot using a tumor DNA dilution series with a known mutation codon 12 Val.
(1=80%, 2=40%, 3=20%, 4=10%, 5=5% and 6=1% tumor cells)

Performance

Workload, time to results, hands-on time, flexibility, DNA input and costs were compared for the different techniques used and are summarized in table 2. The workload and time to results are similar for direct sequencing and SNaPshot. For both techniques it involves, PCR, PCR product purification, either extension or sequencing reaction, second purification step and subsequent run by capillary electrophoresis. The hands-on time post DNA isolation for both techniques is approximately two hours work. The time to results, post DNA extraction, is approximately two days for direct sequencing and one and a half days for SNaPshot around respectively. When using the StripAssay™, hands-on time is about one and a half hours and time to results post DNA extraction, can be half a working day.

DNA input is similar in all three assays tested. Generally, the isolation of DNA from 1 cm^2 tissue is enough to perform several reactions.

Costs for reagents vary from 5 euros per sample for direct sequencing and SNaPshot assay to 80 euros per sample for the StripAssay™ in the Netherlands. However, labor is not included in these prices nor the costs of dedicated laboratory equipment necessary to carry out sequencing and SNaPshot assay.

SNaPshot is the most flexible of the three techniques facilitating the use of multiplex reactions. Direct sequencing does not allow the use of multiplex PCR. The StripAssay™ is a commercial assay; its flexibility is poor and depends on the manufacturer's choice in further development.

Clinical validation

KRAS mutations were found in 107 of the 296 colon cancer samples tested, 36% of the study group. Table 3 shows the frequencies of the different mutations found in these samples. On average, mutation frequencies were in agreement with frequencies published in the COSMIC database (http://www.sanger.ac.uk/genetics/CGP/cosmic/ viewed June 30th, 2010). These results were identical with direct sequencing and with single nucleotide primer extension.

The c.38G>T; p.Gly13Val mutation which is not available in the StripAssay™ was found in one sample from the 296 in this cohort.

Table 2: Evaluation of performance of the three techniques.

	Direct sequencing	SNaP shot	StripAssay™
Workload	Laborious	Laborious	Time sparing
Result interpretation	Time consuming	Easy	Easy
Sensitivity	20%	10%	1%
Quantification	semiquantitative	semiquantitative	Non quantitative
Flexibility	No	Yes	No
Costs	4 euro	4 euro	85 euro*
Assay hands-on time	2 hours	2 hours	1.5 hours
Time to results	2 working days	1,5 working days	1 working day
Special equipment	Sequence facilities	Capillary electrophoresis	Not required

* Costs are estimated costs for reagents (no labour included) in the Netherlands

Table 3: *KRAS* mutation frequencies according to COSMIC database and in colon cancer samples.

Nucleotide mutation	Codon substitution	Aminoacid substitution	Mutation frequencies in the present cohort N (%)	Mutation % according to COSMIC database
c.35 G>T	c12 GGT>GTT	p.Gly12Val	19/107 (18)	22.9
c.35 G>A	c12 GGT>GAT	p.Gly12Asp	33/107 (31)	35
c.35 G>C	c12 GGT>GCT	p.Gly 12Ala	9/107 (8)	6.5
c.34 G>T	c12 GGT>TGT	p.Gly12Cys	9/107 (8)	9
c.34 G>A	c12 GGT>AGT	p.Gly12Ser	6/107 (6)	6.5
c.34 G>C	c12 GGT>CGT	p.Gly12Arg	3/107 (3)	1.3
c.38G>A	c13 GGC>GAC	p.Gly13Asp	26/107 (24)	17.6
c.38 G>T	c13 GGC>GTC	p.Gly13Val	1/107 (1)	0.1
c.37 G>T	c13 GGC>TGC	p.Gly13Cys	1/107 (1)	0.5
c.37 G>C	c13 GGC>CGC	p.Gly13Arg	0	0.3
c.37 G>A	c13 GGC>AGC	p.Gly13Ser	0	0.15
c.38G>C	c13 GGC>GCC	p.Gly13Ala	0	0.1

Discussion

The recent advices from the ASCO and a European expert panel to perform *KRAS* mutation detection prior to therapy with cetuximab in metastatic colorectal cancer [5] and in stage II and III colon cancer [6], respectively, has made the need for a sensitive, flexible, fast and easy to implement in daily practice assay urgent. Therefore, we compared three currently available techniques for implementation in routine diagnostics. The gold standard direct sequencing was compared to "in house" developed SNaPshot and partly to the commercially available StripAssay™.

Several parameters were accounted for including sensitivity, specificity, workload, time to results, hands-on time, flexibility and costs. However, the choice of a technique also depends on other variables such as equipment, expertise and personnel available in a molecular diagnostics laboratory.

In this study, SNaPshot showed to be a very sensitive technique which performed well with paraffin embedded tissues. Without any mutant DNA enrichment strategy before the *KRAS* specific PCR, we obtained reproducible and robust results in the entire cohort of patients tested. All mutations previously obtained with direct sequencing were confirmed with the SNaPShot technique and frequencies agreed with the COSMIC database (table 3). The fully consistent results between SNaPshot and direct sequencing can be explained by the selection of samples. All samples must contain more than 30% tumor cells, which in turn is higher than the detection threshold for both techniques 10 and 20% respectively. Moreover, both techniques compared are performed using different PCR products, but the same DNA extracted from clinical specimens. We know that DNA extraction is a crucial factor for test reproducibility and subsequent possible differences in sensitivity. Workflow is similar to direct sequencing, hands-on time post DNA extraction is approximately two hours whereas time to results after DNA isolation is approximately one and a half working days. In our opinion, the SNaPshot assay has two main advantages when compared to direct sequencing. First, SNaPshot was more sensitive than dideoxy sequencing being able to detect mutations in samples containing 10% tumor cells in a background of wild type cells. Second, this technique is very flexible. It is easily extendible to other *KRAS* mutations and to mutations in other genes like for instance the B*RAF V600E* mutation. This characteristic can be important in the future. With the introduction of more targeted therapies it seems likely that gene mutation detection is going to be a cornerstone in molecular diagnostics. This flexibility

can save diagnostic time and material input, besides reducing costs [15]. However, primer design can be complicated and the use of multiplex reactions could affect sensitivity and therefore this issue should be addressed before implementing it in daily practice.
In our hands, the most sensitive assay was the StripAssay™ based on mutant enriched PCR followed by reverse hybridization. The mutant enriched PCR is based on the clamping of the wild type sequence by PNA nucleotides therefore, only mutant DNA template is amplified. With this technique mutations were detected in samples containing as little as 1% tumor cells in a wild type background. These results are in agreement with previous reports using cell lines [11] where the same sensitivity was found for mutation detection.
Although the hybridization to a specific probe after PCR amplification minimizes the risk of false positive results, one drawback of PNA PCR clamping can be false positivity due to Taq polymerase errors under the clamp depending on the amount of DNA template [16; 17]. Thus, one should be aware of the fact that false positivity is a real concern when using techniques based on PNA PCR clamping. However, in our case, it is difficult to assess whether the false positivity was introduced during the PCR or during sequencing. The fact that clamped PCR products did no hybridize to the StripAssay™ but were found after sequencing, indicates that at least in one sample the error occurred during sequencing. Nevertheless, to minimize the risk of false positivity introduced by Taq polymerase errors, assays should be performed in duplicate and manufacturer's instructions concerning DNA input, should be strictly followed. The latter, might be a difficult issue when using FFPE, since measurement of DNA amount is often unreliable. Furthermore, such a sensitive technique could detect small subpopulations of tumor cells carrying mutant alleles within a majority of wild type tumor cells. Although *KRAS* mutation is generally accepted as an early event in colon carcinogenesis [4], tumor heterogeneity is a known feature [18]. Baldus *et al* [18] have recently reported that mutations are differentially present in different areas of the tumor as well as in positive lymphnodes and metastasis. The clinical relevance of this finding is not fully understood, but it could greatly contribute to difficult therapy decision making. Mutated clones could be preferentially detected with the StripAssay™, while remaining undetectable with standard techniques such as direct sequencing and SNaPshot, even when sufficient tumor cells are present.
Thus, the high sensitivity of the StripAssay™ could be its biggest caveat and one should be very cautious when carrying out such a sensitive assay. It might well be

that even more expertise, more restricted laboratory discipline and special additional precautions are necessary to circumvent false positivity due to sample contamination. Furthermore, it is strongly recommended to confirm StripAssay™ positive samples by either a new StripAssay™ or another assay with a similar analytical sensitivity.

The workflow of the StripAssay™ is easy, the hands-on time is approximately one and a half hours and time to results after DNA isolation is half working day. This assay does not require any dedicated equipment. Thus, results can be obtained within one working day, halving diagnostic time. The price of the StripAssay™ currently commercialized by Vienna Labs (Vienna Labs, Vienna, Austria) is not competing with dideoxy sequencing or the SNaPshot assay in the Netherlands. The costs of mutation detection per sample with the StripAssay™ are approximately 20 fold higher than using direct sequencing or SNaPshot assay; however, labour costs are not included, dedicated equipment is not needed and finally, investment is not necessary for assay development, validation and quality control of reagents. Moreover, the StripAssay™ can be performed in all laboratories without dedicated equipment, whereas for direct sequencing and the SNaPshot technique a sequence capacity or a capillary electrophoresis machine are mandatory.

Such low detection thresholds are not necessary in colon cancer molecular diagnostics. In general colon cancer samples contain more than 20% tumor cells. Nevertheless, for other tumor types such as neoadjuvantly treated rectal cancer without available biopsies and for lung cancer biopsies and cytology, high sensitivity is an important issue and sensitive techniques like the StripAssay™ might be clinically valuable.

Other available techniques for *KRAS* mutation detection can also reduce workload, prices, time to results and sensitivity. HRM is recently described as a good alternative screening method [7]. It is rapid, sensitive and accurate [19]. By screening all samples with HRM, only aberrant samples need to be further analyzed to determine the underlying mutation, thereby decreasing sequencing workload. However, costs might increase, when no dedicated technology for HRM is present and must be additionally bought. Pyrosequencing is a sensitive, rapid and less laborious technique that can be a good alternative to direct sequencing. An advantage of pyrosequencing is that it is a quantitative assay which does not need PCR product manipulation diminishing contamination risk [8]. Finally, real time allelic discrimination could also be a good alternative for direct sequencing because of the rapidity and high sensitivity of the technique; however, the difficulty of multiplexing and the similarity between the

probes lead to higher DNA input and a high risk of decreased specificity due to cross reactivity of the different probes [10].

When considering all aspects, we conclude that for colon cancer diagnostics, in which, sensitivity is generally not an issue and when capillary electrophoresis facilities are already available, SNaPshot can be as valuable as direct sequencing. Workflow, time to results, hands-on time and costs do not vary much between both techniques. However, the multiplex possibilities of the SNaPshot can reduce DNA input, costs and workload. Thus SNaPshot is a good alternative for direct sequencing for *KRAS* mutation detection in colon cancer patients in daily diagnostic practice. However, when sensitivity is an important issue such as in the case of lung cytology samples, or for small laboratories without dedicated equipment, highly sensitive techniques like the StripAssay™ should be considered due to its high sensitivity, rapidity and ease to perform. Nevertheless, one should be aware of the false positivity risks of such a technique and perform assays in duplicate to avoid false positives.

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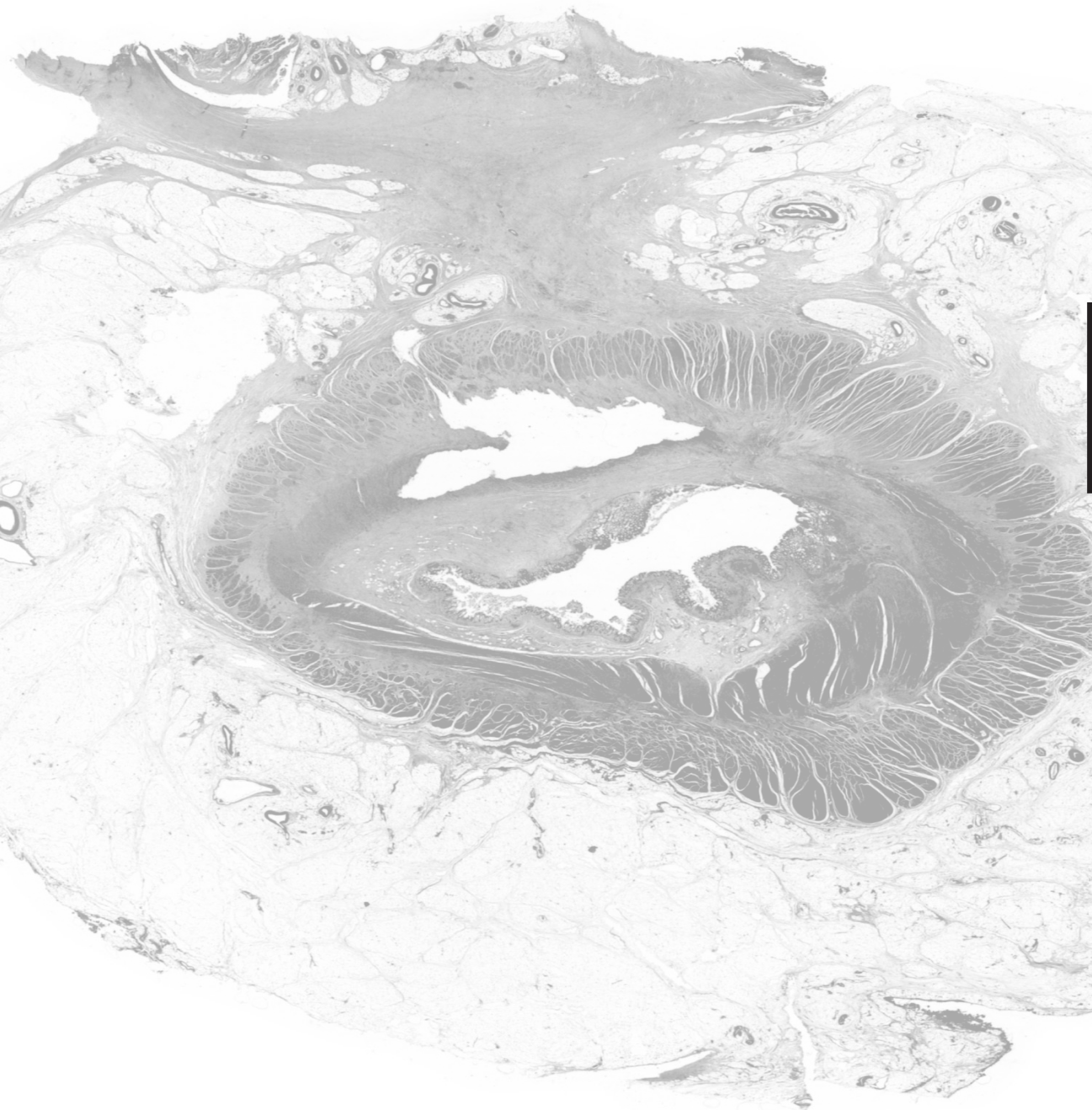

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Concluding remarks and future perspectives

Concluding remarks and future perspectives

The survival rates of patients with metastatic colorectal cancer have improved significantly due to the recent introduction of novel therapies. Moreover, the use of cetuximab, panitumumab and bevacizumab has accelerated the implementation of molecular testing in colon cancer diagnostics. Indeed, *KRAS* mutation detection in stage IV colorectal cancer has become standard practice in many pathology laboratories and other markers like *BRAF* V600E and *PIK3CA* will probably follow in the near future.

However, molecular characterization is currently used only in stage IV disease and not in earlier disease stages. Therefore, stage II and III are less well characterized at the molecular level, forming a rather heterogeneous disease group. Several parameters such as tumor localization, mismatch repair (MMR) status or tumor histology influence clinical behavior but are often not taken into account when defining clinical subsets.

Hence, this intertumor heterogeneity, together with intratumor heterogeneity and tumor plasticity are probably reasons for the discrete improvements in survival rates in these stages[1] and the somewhat disappointing results of some of these novel clinical trials of the last decade[2-4].

In stage II and III colon cancer, the identification of patients at risk of relapse, due to therapy resistance or to tumor intrinsic aggressiveness, is needed in order to improve disease management and outcome. Therefore, the main focus of this thesis was to identify molecular prognostic and predictive markers of response to therapy in stage II and III sporadic colon cancer. Predictive markers can identify patients who are not likely to respond to a certain chemotherapeutic drug, helping to decrease unnecessary exposure to that particular drug and thus toxicity. On the other hand, prognostic markers will identify patients with a poor natural history of their disease who will probably benefit from adjuvant chemotherapy or even from a more aggressive form of therapy than recommended by the guidelines.

Pharmacogenetics & Predictive Markers

Since the mid-nineties the therapy guidelines for colon cancer management recommend the use of adjuvant chemotherapy after curative intended surgery for all patients with stage III colon cancer. This recommendation improved colon cancer patients' survival. Risk of cancer related death in stage III patients was reduced in 29% (CI 13-42%) with 5-FU monotherapy[5]. Combination of 5-FU with oxaliplatin, administered since 2005, reduced the risk of cancer related death with another 20%[6]. Despite this significant improvement in patient survival, a large percentage of patients apparently still do not experience any benefit from the treatment.

We studied eight polymorphisms in genes coding for proteins involved in the metabolism of 5-FU and oxaliplatin such as the *thymidylate synthase* (*TYMS*), *thymidine phosphorilase* (*TYMP*), *dehydropyrimidine dehydroxilase* (*DYMP*), *orotate phosphoribosyltransferase* (*OPRT*), *glutathion S transferase Pi* (*GSTPI*), *excision repair cross complementing group 1* (*ERCC1*) and *excision repair cross complementing group 2* (*ERCC2*) genes in stage III sporadic colon cancer patients. None of the polymorphisms studied was found to be a reliable marker predictive of therapy response in stage III disease.

These markers have been extensively studied by us and others, not only at the DNA level and in colon cancer but also at expression level (mRNA and protein) and in other types of cancer. Their value as predictive markers remains elusive because of conflicting results[7-71]. However, research groups did find certain genotypes (alone or combined) of the cited genes predictive of therapy response in colon cancer patients or indicative of therapy toxicity[7-9,12,23-25,37,71].

The contradictory and inconclusive results might be explained by the retrospective character of the majority of the studies and the diversity of molecular techniques used. Furthermore different SNPs and genotype combinations were tested. On top of this, the results of functional experiments assessing the effect of a certain SNP in protein function and expression turned out to be contradictory as well[67,72]. This all makes the biological interpretation of the results complicated and probably unreliable. Moreover, most of the studies examined a heterogeneous population of patients including different disease stages, and differently located cancers (left-, right-sided or rectum). All these factors might give rise to the different results. Finally, even studies reporting positive relations between certain genotypes and disease outcome or therapy toxicity,

advocate for validation in prospective trials or larger cohorts before implementation in clinical practice.

Therefore, based on the existing literature and our experience, we conclude that in order to discard or implement such genetic markers in clinical practice, two types of studies are mandatory. Firstly, functional studies reporting the effect of SNPs on gene expression, protein function etc. are essential to determine which SNPs are likely to be relevant in pharmacogenetics. Secondly well-designed association studies, within prospective clinical trials are needed. Prospective clinical trials fulfill several criteria like large cohorts of patients that are carefully documented and homogeneously treated. Indeed, this approach has been used for reporting associations between toxicity and SNPs[10,73] but less frequently for therapy response[51]. Another possibility is a retrospective study with an exploratory and a validation cohort. However, to study therapy response and toxicity, patients should have been equally treated and clinical course should have been carefully documented. In case of an exploratory and a validation cohort these are frequently not equally treated because of differences in disease management depending on location and time of diagnosis.

Tan *et al* recently published the results of a clinical trial with rectal carcinoma patients. These patients were randomized between standard 5-FU based chemoradiotherapy and alternative 5-FU combined with irinotecan chemoradiation, on the base of a *TYMS* genotype. The authors concluded that classification of patients based on their genotype and subsequent variation of the therapy was feasible and that therapy results improved with this pharmacogenetic approach[74]. The latter trial constitutes a first step towards the incorporation of molecular pharmacogenomic testing in personalizing therapies in early stages of colon cancer. However, it also raises the question whether there is enough scientific evidence for these kinds of trials.

Somatic Mutations and Prognostic Markers

Given the enormous expansion of targeted therapies and their price coming with it, prognostic/predictive markers are essential for accurate patient's classification and disease management. In addition, the molecular classification of patients and their tumors will contribute to more homogeneous study groups increasing the probability of reliable results and improvements in colon cancer therapy.

Prognostic markers are useful for a more accurate classification of patients and can identify different prognostic subgroups as seen for the *BRAF* V600E mutation. The latter mutation not only identifies patients with a poor prognosis independently of disease stage and even MMR status, but it also seems to characterize a type of tumor with an own genomic profile that is different than double wild type tumors.

However, not all mutations have such a clear association with prognosis like *BRAF* V600E. We show in this thesis that the mutation in exon 20 of the *PIK3CA* gene has only prognostic value in stage III disease and not in stage II. Moreover, we also report that gene-gene interactions can affect the prognostic effects of certain makers. This is the case of *TP53* inactivation which prognostic effects are greatly affected by the differential expression of the *CSNK1A1* gene. Thus, although very complex, gene-gene interactions also need to be studied within the scope of prognostic markers research.

In conclusion, molecular analysis of cancer cells can potentially aid to classify tumors more accurately and to manage patients accordingly. However, prognostic biomarkers need to be integrally analyzed to be able to explore genetic interactions and subtle molecular relations. Therefore, combined genetic, genomic, epigenetic and expression studies should be carried out. Likewise, basic functional research is essential to learn more about genetic interactions and to be able to correctly interpret data obtained from new techniques like SNP arrays or next generation DNA/RNA sequencing.

Future Perspectives

To decrease colorectal cancer death in the future, two complementary approaches are necessary; on one hand, disease prevention and early diagnosis and on the other hand accurate disease classification should be established for personalized therapy.

Disease prevention

By implementing screening programs for colorectal cancer, malignant tumor development can be prevented by excising premalignant polyps and cancer can be diagnosed at earlier stages like stage I/II when surgery is still curative. Indeed, several Western countries are implementing population based screening programs. The expectation, in The Netherlands, is to reduce colorectal cancer incidence and prevent mortality in 2400 patients per year out of the current 10 000 and therefore reduce treatment costs[75].

Molecular disease classification

The second approach consists of the identification, validation and general implementation of molecular signatures identifying colon cancer subgroups.

At this moment, all colon cancer patients with stage III and high risk stage II are treated equally without taking into account tumor molecular signatures. Recently, two colon cancer gene expression signatures associated with disease recurrence and poor prognosis in early stages have been published[76,77]. Although they have not been approved for clinical use yet, they represent one step forward in the use of molecular profiling in colon cancer classification.

In the near future standard molecular stratification of patients and tumors should be able to define subgroups of patients leading to personalized treatment protocols.

A problem herewith is intratumor heterogeneity as well as tumor plasticity. Intratumor heterogeneity has been recognized for a long time now by surgeons, oncologists, pathologists and molecular biologists. Tumors may contain multiple clones that do not necessarily share the same molecular signatures or phenotypes. The different clones in a particular tumor evolve in time depending on tumor environmental influences like growth factors, hypoxia, inflammation, immune responses, stroma composition, et cetera. The study of these topics is technically challenging and difficult to solve and these subjects are therefore underrepresented in the literature[78,79].

With the introduction of targeted therapies in cancer management tumor heterogeneity and plasticity have become even more important. These therapies target strategically chosen genes with activating mutations, based on the so-called oncogene addiction model. According to this model, cancer cells become dependent of certain activating mutations in key molecules in cell division, cell survival and signaling pathways [80]. Cancer cells can circumvent the blocking of signaling pathway by acquiring novel mutations or switching to other pathways, thereby becoming resistant to a particular therapy. This adaptive capacity of the tumor is probably responsible for the relatively rapid relapses after treatment with targeted therapies seen in clinical practice. Moreover, it is currently unknown what is the minimum percentage of resistant or sensitive cells in order to consider a tumor resistant or sensitive for a given therapy [81]. Thus, the clinical consequences of intratumor heterogeneity need to be further investigated as it is now technically more feasible[79,82].

Molecular pathology enabling the molecular classification of tumors and molecular biomarker determination in cancer diagnostics already plays an important role in daily clinical oncologic practice. However, put into perspective, a relatively very small proportion of molecular markers makes it eventually to daily clinical practice. In the nearby future and derived from the use of new technologies, molecular diagnostics will probably play an essential role in tumor classification. Therefore, specific training of future pathologists in the field of molecular diagnostics is pivotal in order to ensure an effective interplay between oncologists, pathologists and molecular biologists, leading to patient tailored therapy.

Besides, a vivid debate is taking place in the Netherlands about the implemetation of molecular diagnostics in pathology laboratories. At the present time, it is not legaly regulated which laboratory can carry out molecular diagnostics; both academic and non academic centers perform molecular diagnostics in pathology. However, the level of complexity is rapidly increasing, the development of new tests is expensive and specific expertise and knowledge are mandatory to interpret results. Thus, to ensure high quality, competitive prices and ongoing technological research and innovation, expertise and technologies should be, in our opinion centralized.

Molecular prognostic markers or molecular tumor signatures will aid to classify colon cancer patients more accurately in order to improve disease management and patient outcome. These molecular signatures could be a complement to decision making tools for chemotherapy choice and even improve these tools. Molecular predictive

markers will help reduce cancer treatment toxicity of unnecessary therapy regimens. Collaborative studies to reach enough statistical power are mandatory to identify small subgroups of patients behaving differently clinically. Integral typing of these samples i.e. at a genetic, genomic, regulatory, epigenetic and expression level, mRNA, miRNA and protein levels, is recommended. Basic functional research is mandatory to make biological sense of data obtained from whole genome analyses. Finally, elucidating the role of intratumor heterogeneity and plasticity is an important challenge to understand tumor biology and really accomplish personalized therapy in the future.

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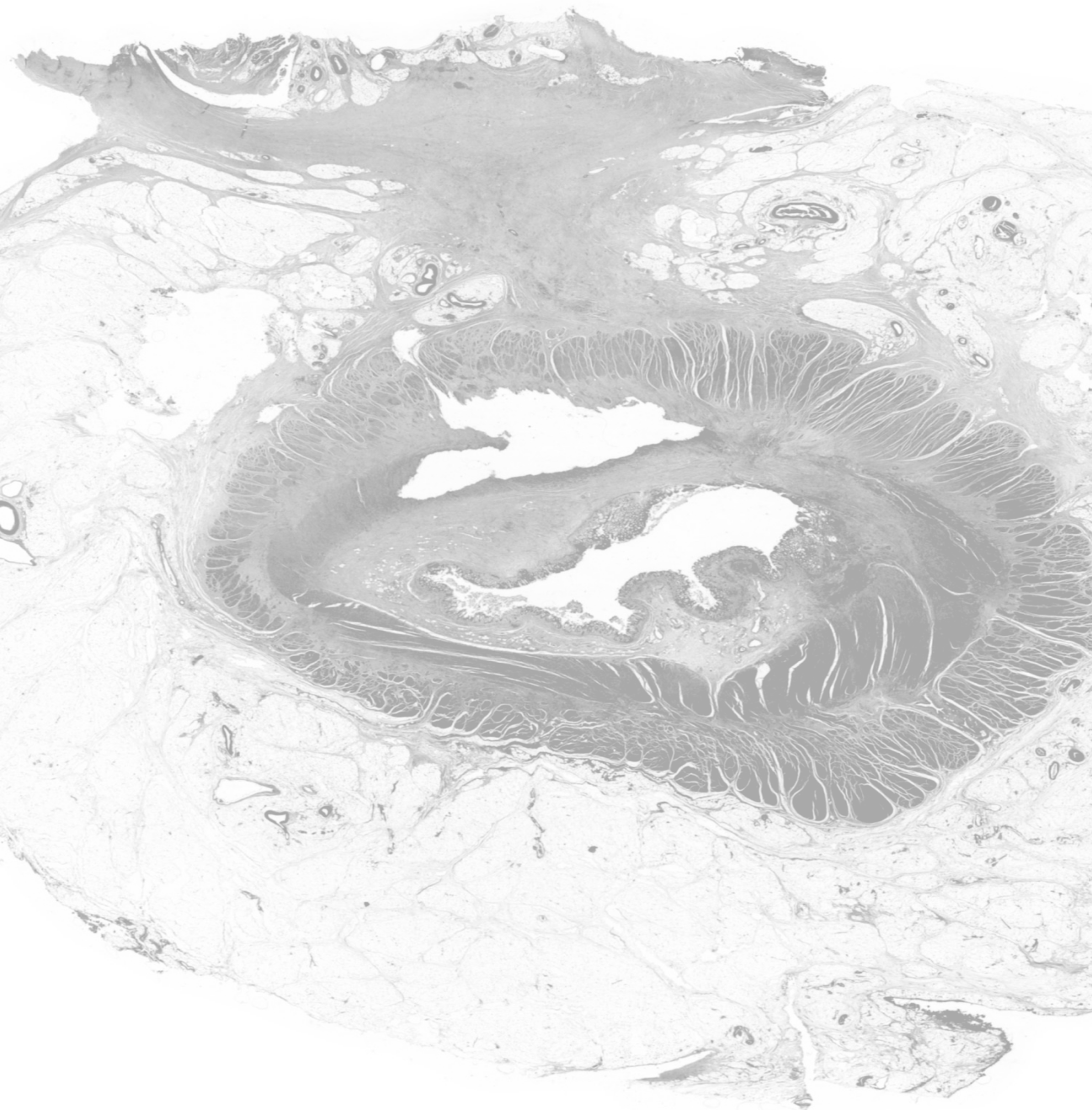

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Samenvatting

Colon carcinoom is het derde meest voorkomende type kanker in de Westerse wereld. In Nederland worden jaarlijks circa tienduizend patiënten gediagnosticeerd met colon kanker en ongeveer drie tot vier duizend patiënten overlijden aan deze ziekte. Vooral als de ziekte uitgezaaid is, is de prognose zeer slecht. In stadium II is de ziekte alleen aanwezig in de dikke darm, terwijl in stadium III, de ziekte tot in de lymfeklieren is verspreid. In Nederland krijgen patiënten met stadium III ziekte, na chirurgie, chemotherapie om de kansen op overleving te vergroten. Stadium II patiënten krijgen alleen chemotherapie als ze hoog risico ziekte hebben. Hoog risico wordt gedeffinieerd als een slecht gedifferentieerde tumor, diep invaderende tumor (T4), obstructie of perforatie van de darmwand of als minder dan 10 lymfklieren zijn onderzocht. De vijf jaar overleving varieert tussen 80% in stadium II tot 65% in stadium III. Deze cijfers zijn nogal teleurstellend voor een ziekte die alleen lokale tot regionale spreiding kent. De identificatie van markers van respons op chemotherapie en prognose zijn derhalve essentieel in deze twee stadia om patienten beter te kunnen classificeren en te behandelen, om de overlevingkansen te vergroten.

Het onderzoek beschreven in dit proefschrift kent twee hoofddoelen; ten eerste het identificeren van moleculaire markers van chemotherapie respons in stadium III colon carcinoom, ook predictieve markers genoemd en ten tweede het bepalen van de invloed op overleving van een aantal genetische markers in stadium II en III colon carcinoom, in andere woorden prognostische markers.

Hoofdstuk 2, 3 en 4 focussen op farmacogenetica, oftewel het bestuderen van DNA variatie in genen betrokken bij het metabolisme van chemotherapeutica en/of bij het herstel van de schade die deze geneesmiddelen veroorzaken. Voor de behandeling van colon carcinoom worden 5-Fluorouracil en oxaliplatin gebruikt. De effecten van DNA variatie in: Thymidylate synthase (*TYMS*), Thymidylate phosphorylase (*TYMP*), Dihydropyrimidine Dehydrogenase (*DPYD)*, Ororate Phosphorybosyl Transferase *(OPRT*) op overleving is onderzocht in een cohort van patienten met stadium III colon carcinoma. Uit onze resultaten blijkt dat geen van de onderzochte DNA polymorfismen een effect heeft op overleving. Daarnaast is het effect op overleving, van variatie in DNA van genen betrokken in het herstel van schade veroorzaakt door oxaliplatin, zoals *ERCC1, ERCC2* en in het metabolisme van oxaliplatin zoals *GSTPI*, onderzocht. Wederom blijkt de genetische variatie geen effect te hebben op overleving.

In hoofdstuk 5, 6, 7 en 8 wordt de waarde van mutaties in bepaalde genen als prognostische markers onderzocht. Deze genen zijn allemaal betrokken in het ontstaan van colon kanker of bij de progressie van de ziekte.
Het blijkt dat de V600E *BRAF* mutatie een groep van tumoren identificeert met een zeer aggressief ziektebeloop. Daarnaast leveren we bewijs dat deze tumoren verschillen op genomisch niveau van tumoren zonder die mutatie.
Tevens identificeren mutaties in het kinase domain van het *PIK3CA* gen een kleine subgroep van stadium III patiënten met een slechter beloop van de ziekte. Opvallend is dat dezelfde mutatie geen effecten in overleving blijkt te hebben in stadium II van de ziekte. Evenmin hebben mutaties in andere regio's van het gen effect op overleving.
Verder wordt in dit proefschrift de rol van p53 als prognostische marker in colon carcinoom beschreven. Hieruit blijkt een ander gen, namelijk caseine kinase 1 alpha subunit 1 (*CSNK1A1),* een belangrijke rol te spelen. Hoge expressie niveaus van het *CSNK1A1* gen herstellen de negatieve effecten in overleving van een niet actief p53 eiwit. Lage niveaus van *CSNK1A1* en een niet actief p53 veroorzaken echter een zeer slechte overleving.
Concluderend, moleculaire pathologie voor de moleculaire classificatie van tumoren in het algemeen en colon carcinoom in het bijzonder zal in de nabije toekomst een belangrijke rol spelen om de therapie van patienten beter te bepalen.

Summary

There is an urgent need for predictive and prognostic markers in early stages colon carcinoma to be able to elucidate whether a patient is going to respond to therapy or not and also to be able to offer personalized treatment.
In this study, we aimed to identify predictive markers of therapy response in stage III disease and prognostic markers in stage II and III colon carcinoma.
In chapters 2, 3 and 4, the focus lies on pharmacogenomics with the aim to identify predictive markers. As colon cancer is treated with 5-Fluorouracil and oxaliplatin, the effect of DNA polymorphisms in genes involved in the metabolism of these drugs and in DNA damage repair caused by oxaliplatin on disease free survival was studied.
Therefore, several polymorphisms in the following genes were tested; Thymidylate synthase (*TYMS)*, Thymidylate phosphorylase (*TYMP*), Dihydropyrimidin Dehydrogenase *(DPYD*) and Ororate Phosphorybosyl Transferase *(OPRT*) together with Glutation S Transferase Pi (*GSTPI*), Excision Repair Cross-Complementation group one (*ERCC1*) and two (*ERCC2)*. We concluded that none of the SNPs studied seemed to have effects on the disease free survival of stage III colon cancer patients. Thus, none of the studied SNPs was a reliable predictive marker of 5-FU or oxaliplatin response.
In chapter 5, the focus is placed on the identification of molecular prognostic markers in stages II and III. The *BRAF* V600E mutation, mutations in codons 12 and 13 of *KRAS* and microsatellite instability were studied. *BRAF* V600E mutation conferred a poorer prognosis to colon cancer patients independently of microsatellite instability, *KRAS* mutational state, age, gender, T and N stage.
The value of mutations in other genes involved in signal transduction like *PIK3CA* is described in chapter 6. Mutations in the helical and in the kinase domain of this protein have different effects on survival. Moreover, while mutation in the kinase domain of *PIK3CA* in stage III disease conferred a very poor prognosis, the same mutation in stage II disease did not affect survival. Mutations in the helical domain did not affect survival in stage II nor in stage III disease.
In chapter 7, we try to unravel the role of p53 in prognosis of colon cancer. *TP53* seems to be a haploinsufficient tumor suppressor gene implying that patients losing one allele and retaining the wild type allele are at risk of developing a tumor albeit with a favorable prognosis. Moreover, we also illustrate the importance of studying gene-gene interactions, as we found that expression of caseine kinase 1 alpha subunit 1

modifies greatly the effects of *TP53* on survival. High *CSKN1A1* expression counteracts the negative effects of a not functional p53 protein, whereas low *CSNK1A1* expression decreases even more survival of patients with not functional p53.

In chapter eight, the genomic differences between *BRAF* mutant and double wild type tumors are described. *BRAF* mutated tumors seemed to be genomically more instable than double wild type tumors. These tumors also show specific genomic alterations that differ from double wild type tumors.

Finally, in chapter nine, the challenges that diagnostic tests have to deal with before being implemented in daily clinical practice are described, taking *KRAS* mutation analysis as an example.

Resumen

El adenocarcinoma de colon es el tercer tipo de cáncer mas frecuente en el mundo occidental. A pesar del gran número de investigaciones sobre el cáncer de colon, las esperanzas de vida de estos pacientes no han mejorado mucho en los últimos años. Este proyecto se centra en los estadios II y III en los que la enfermedad está localizada en el colon o ha invadido ganglios linfáticos regionales. El tratamiento actual de estos pacientes es cirugía seguida de quimioterapia adyuvante, en el caso de que haya expansión linfática. Siguiendo estas directivas, hay pacientes que no responden a la quimioterapia, hay pacientes que no la necesitan puesto que la cirugía podria ser considerada curativa y hay pacientes que se beneficiarían de la quimioterapia pero no la reciben. Por eso, los dos objetivos fundamentales de este proyecto eran identificar marcadores moleculares de respuesta a la quimioterapia en pacientes con estadio III por un lado y por otro identificar marcadores pronostico en estadio II y III para clasificar a los pacientes más adecuadamente.

Los tres primeros capítulos de esta tesis se centran en la farmacogenetica y la identificacion de marcadores predictivos. La farmacogenetica estudia el efecto de variaciones o polimorfismos en el ADN de genes que codifican proteinas involucradas en el metabolismo de determinados farmacos, en la supervivencia de los pacientes tratados con estos farmacos. Este trabajo se centra en proteinas involucradas en el metabolismo del 5-Fluorouracilo como la timidilato sintetasa, timidilato fosforilasa, dihidropirimidina deshidrogenasa y el ororato fosforibosil transferasa por un lado y por el otro en el metabolismo del oxaliplatino como ERCC1, ERCC2 y la glutation S transferasa Pi. Los resultados obtenidos indican que ninguno de los polimorfismos estudiados es un buen marcador predictivo de respuesta al tratamiento.

Por otro lado, también se estudio el valor pronóstico de mutaciones en conocidos genes relacionados con el cáncer. De este modo en el capitulo cinco se expone que la mutación V600E en el gen *BRAF* caracteriza a un grupo de pacientes con mal pronóstico independientemente de la localización del tumor o del estadio del mismo. El capitulo seis describe que mutaciones en el codón 20 del gen *PIK3CA* también afectan negativamente la supervivencia de pacientes con estadio III de la enfermedad. Este efecto negativo no se ve en pacientes con estadio II. Además, también se ha estudiado el valor de p53 en el pronóstico de esta enfermedad como se expone en el capitulo siete. Estos resultados ilustran la importancia de la relación entre genes. El efecto

pronostico negativo de un p53 inactivo se ve contrarrestado por la elevada expresión del gen caseina kinasa 1 alfa subunidad 1 (*CSNK1A1)*. Del mismo modo, el pronóstico de pacientes en los que el tumor tenga un p53 inactivo y baja expresión de *CSNK1A1* es tremendamente desfavorable.

Por último, en el capitulo ocho, presentamos las diferencias a nivel genómico de tumores con la mutación V600E en el gen *BRAF* y tumores sin esta mutación. Demostramos que los tumores con la citada mutación son cromosómicamente más inestables y que además tienen aberraciones focales distintas de los tumores sin la citada mutación.

Curriculum Vitae

Arantza Fariña Sarasqueta was born on December 4th, 1971 in Bilbao, Spain. She graduated as a medical doctor by the University of the Basque Country (Universidad del Pais Vasco, UPV/EHU). Thereafter, she left to the Netherlands. In 2001, she graduated as a medical biologist by the Free University Amsterdam (Vrije Universiteit, VU). She started a research project at the department of pediatric oncology at the Sophia Children Hospital in Rotterdam, on molecular prognostic factors in childhood T cell acute lymphoblastic leukemia. In 2008, she started the present PhD project on prognostic and predictive molecular markers in colon cancer. She works currently as a resident at the Pathology department of the Leiden University Medical Centre.

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