CHAPTER 10

Summary, general discussion, conclusions and future directions

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

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

PART 1 BIOMARKERS OF APOPTOSIS AND PROLIFERATION

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

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

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

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

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

for clinical application the patient's tumor microsatellite status should therefore always be taken into account.
In **Chapter 5** we validated the prognostic quality of the combined analysis of proliferation and apoptosis when both determined with biochemical assays, CKD1SA reflecting tumor cell proliferation and caspase-3 activity reflecting tumor cell apoptosis, in a cohort of stage II colon cancer patients taking into account the patients tumor microsatellite status. In this cohort, low levels of tumor cell CKD1 SA and high levels of casapse-3 activity were independent predictors of better DFS (CDK1 SA HR 9.6, p-value 0.029 and caspase-3 activity HR 0.5, p-value 0.03). The combined results of these assays, correctly classified 87.5% of the stage II MSS patient as high risk of developing distant metastasis.
In summary, we demonstrated the clinical importance of combined analysis of the level of tumor cell apoptosis and proliferation in chapter 3 and the use of a biochemical assay reflecting tumor proliferation and apoptosis in chapter 4. Furthermore, in chapter 5 the results of our combined analysis were compared to a subset of the ASCO criteria, and showed to perform equally with respect to test specificity and sensitivity (12). Thus, we were able to demonstrate that the use of our apoptosis/proliferation parameter, and taking into account the tumor microsatellite status, is of additive value in the high-risk patient identification.

PART 2 TUMOR-IMMUNE INTERACTIONS

The immune system has been attributed an important role in controlling tumor growth and metastasis (33–36). New insight emerging from the growing field of cancer research have led to the addition of two hallmarks, gained during tumor development, to the six biological capabilities tumor cells have acquired in the multistep process of malignant transformation as originally described by Hanahan and Weinberg in 2000 (20,37). One of these two emerging hallmarks is the capability of tumor cells to evade immune destruction. It has become clear that tumor cells during their transformation, and in interaction with their host, create a so-called tumor micro-environment (38). One of the characteristics of this tumor micro-environment is interaction of tumor cells with the immune system. The tumor immune surveillance hypothesis which postulates that the immune system can identify cancerous cells and eliminate them, probably usually before they can cause harm is based on this observed interaction (39). Based on this hypothesis studying tumor immune-interactions provides us not only with new biomarkers as described in chapter 8 and 9, it also provides a possibility to develop new treatment strategies for the high-risk patient cohort.
One of the new immune-based treatment strategies, the use of vaccines, exploits activation of the patient's immune system as a therapeutic modality. In **Chapter 6** we provide a review of the literature on clinical trials in which both tumor cell-derived vaccines as well tumor antigen-derived vaccines are deployed. To eliminate tumor cells a vaccine needs to induce a robust tumor-specific immune

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

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

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

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

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

CONCLUSIONS AND FUTURE DIRECTIONS

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

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

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

REFERENCE LIST

(1) Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 2010;127(12):2893-2917.

(2) Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011;61(2):69-90.

(3) Van CE, Borras JM, Castells A et al. Improving outcomes in colorectal cancer: Where do we go from here? Eur J Cancer 2013.

(4) Moertel CG, Fleming TR, Macdonald JS et al. Levamisole and fluorouracil for adjuvant therapy of resected colon carcinoma. N Engl J Med 1990;322(6):352-358.

(5) Quirke P, Cuvelier C, Ensari A et al. Evidence-based medicine: the time has come to set standards for staging. J Pathol 2010;221(4):357-360.

(6) Gunderson LL, Jessup JM, Sargent DJ, Greene FL, Stewart AK. Revised TN categorization for colon cancer based on national survival outcomes data. J Clin Oncol 2010;28(2):264-271.

(7) Gray R, Barnwell J, McConkey C, Hills RK, Williams NS, Kerr DJ. Adjuvant chemotherapy versus observation in patients with colorectal cancer: a randomised study. Lancet 2007;370(9604):2020-2029.

(8) Moertel CG, Fleming TR, Macdonald JS et al. Intergroup study of fluorouracil plus levamisole as adjuvant therapy for stage II/Dukes' B2 colon cancer. J Clin Oncol 1995;13(12):2936-2943.

(9) Andre T, Boni C, Navarro M et al. Improved overall survival with oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment in stage II or III colon cancer in the MOSAIC trial. J Clin Oncol 2009;27(19):3109-3116.

(10) Kuebler JP, Wieand HS, O'Connell MJ et al. Oxaliplatin combined with weekly bolus fluorouracil and leucovorin as surgical adjuvant chemotherapy for stage II and III colon cancer: results from NSABP C-07. J Clin Oncol 2007;25(16):2198-2204.

(11) Efficacy of adjuvant fluorouracil and folinic acid in colon cancer. International Multicentre Pooled Analysis of Colon Cancer Trials (IMPACT) investigators. Lancet 1995;345(8955):939-944.

(12) Benson AB, III, Schrag D, Somerfield MR et al. American Society of Clinical Oncology recommendations on adjuvant chemotherapy for stage II colon cancer. J Clin Oncol 2004;22(16):3408-3419.

(13) Dutch Surgical Colorectal Audit. Dutch Surgical Colorectal Audit Jaarrapportage 2010 Uitkomst van zorg registratie; transparantie, uitkomst van zorg en kwaliteit. Online . 2010. Ref Type: Abstract

(14) Fulda S, Debatin KM. Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. Oncogene 2006;25(34):4798-4811.

(15) Degterev A, Boyce M, Yuan J. A decade of caspases. Oncogene 2003;22(53):8543-8567.

(16) de HP, de Bruin EC, Klein-Kranenbarg E et al. Caspase-3 activity predicts local recurrence in rectal cancer. Clin Cancer Res 2007;13(19):5810-5815.

(17) de Bruin EC, van de Velde CJ, van de Pas S et al. Prognostic value of apoptosis in rectal cancer patients of the dutch total mesorectal excision trial: radiotherapy is redundant in intrinsically high-apoptotic tumors. Clin Cancer Res 2006;12(21):6432-6436.

(18) Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 1992;119(3):493-501.

(19) Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100(1):57-70.

(20) Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144(5):646-674.

(21) Buglioni S, D'Agnano I, Cosimelli M et al. Evaluation of multiple bio-pathological factors in colorectal adenocarcinomas: independent prognostic role of p53 and bcl-2. Int J Cancer 1999;84(6):545-552.

(22) Knosel T, Emde A, Schluns K et al. Immunoprofiles of 11 biomarkers using tissue microarrays identify prognostic subgroups in colorectal cancer. Neoplasia 2005;7(8):741-747.

(23) Michael-Robinson JM, Reid LE, Purdie DM et al. Proliferation, apoptosis, and survival in high-level microsatellite instability sporadic colorectal cancer. Clin Cancer Res 2001;7(8):2347-2356.

(24) Skacel M, Skilton B, Pettay JD, Tubbs RR. Tissue microarrays: a powerful tool for high-throughput analysis of clinical specimens: a review of the method with validation data. Appl Immunohistochem Mol Morphol 2002;10(1):1-6.

(25) Jonges LE, Nagelkerke JF, Ensink NG et al. Caspase-3 activity as a prognostic factor in colorectal carcinoma. Lab Invest 2001;81(5):681-688.

(26) Leers MP, Kolgen W, Bjorklund V et al. Immunocytochemical detection and mapping of a cytokeratin 18 neo-epitope exposed during early apoptosis. J Pathol 1999;187(5):567-572.

(27) Soini Y, Paakko P, Lehto VP. Histopathological evaluation of apoptosis in cancer. Am J Pathol 1998;153(4):1041-1053.

(28) Daidone MG, Silvestrini R. Prognostic and predictive role of proliferation indices in adjuvant therapy of breast cancer. J Natl Cancer Inst Monogr 2001;(30):27-35.

(29) Michels JJ, Marnay J, Delozier T, Denoux Y, Chasle J. Proliferative activity in primary breast carcinomas is a salient prognostic factor. Cancer 2004;100(3):455-464.

(30) van Nes JG, Smit VT, Putter H et al. Validation study of the prognostic value of cyclin-dependent kinase (CDK)-based risk in Caucasian breast cancer patients. Br J Cancer 2009;100(3):494-500.

(31) Kim SJ, Nakayama S, Miyoshi Y et al. Determination of the specific activity of CDK1 and CDK2 as a novel prognostic indicator for early breast cancer. Ann Oncol 2008;19(1):68-72.

(32) Roth AD, Tejpar S, Delorenzi M et al. Prognostic role of KRAS and BRAF in stage II and III resected colon cancer: results of the translational study on the PETACC-3, EORTC 40993, SAKK 60-00 trial. J Clin Oncol 2010;28(3):466-474.

(33) Menon AG, Janssen-van Rhijn CM, Morreau H et al. Immune system and prognosis in colorectal cancer: a detailed immunohistochemical analysis. Lab Invest 2004;84(4):493-501.

(34) Halvorsen TB, Seim E. Association between invasiveness, inflammatory reaction, desmoplasia and survival in colorectal cancer. J Clin Pathol 1989;42(2):162-166.

(35) Murphy J, O'Sullivan GC, Lee G et al. The inflammatory response within Dukes' B colorectal cancers: implications for progression of micrometastases and patient survival. Am J Gastroenterol 2000;95(12):3607-3614.

(36) Naito Y, Saito K, Shiiba K et al. CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal

cancer. Cancer Res 1998;58(16):3491-3494.
(37) Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100(1):57-70.
(38) Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144(5):646-674.
(39) BURNET M. Cancer: a biological approach. III. Viruses associated with neoplastic conditions. IV. Practical applications. Br Med J 1957;1(5023):841-847.
(40) Speetjens FM, Kuppen PJ, Welters MJ et al. Induction of p53-specific immunity by a p53 synthetic long peptide vaccine in patients treated for metastatic colorectal cancer. Clin Cancer Res 2009;15(3):1086-1095.
(41) Russo A, Bazan V, Iacopetta B, Kerr D, Soussi T, Gebbia N. The TP53 colorectal cancer international collaborative study on the prognostic and predictive significance of p53 mutation: influence of tumor site, type of mutation, and adjuvant treatment. J Clin Oncol 2005;23(30):7518-7528.
(42) Menon AG, Morreau H, Tollenaar RA et al. Down-regulation of HLA-A expression correlates with a better prognosis in colorectal cancer patients. Lab Invest 2002;82(12):1725-1733.
(43) Speetjens FM, de Bruin EC, Morreau H et al. Clinical impact of HLA class I expression in rectal cancer. Cancer Immunol Immunother 2008;57(5):601-609.
(44) de Kruijf EM, van Nes JG, Sajet A et al. The predictive value of HLA class I tumor cell expression and presence of intratumoral Tregs for chemotherapy in patients with early breast cancer. Clin Cancer Res 2010;16(4):1272-1280.
(45) Curiel TJ, Coukos G, Zou L et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 2004;10(9):942-949.
(46) Hiraoka N, Onozato K, Kosuge T, Hirohashi S. Prevalence of FOXP3+ regulatory T cells increases during the progression of pancreatic ductal adenocarcinoma and its premalignant lesions. Clin Cancer Res 2006;12(18):5423-5434.
(47) Gao Q, Qiu SJ, Fan J et al. Intratumoral balance of regulatory and cytotoxic T cells is associated with prognosis of hepatocellular carcinoma after resection. J Clin Oncol 2007;25(18):2586-2593.
(48) Pepe MS, Etzioni R, Feng Z et al. Phases of biomarker development for early detection of cancer. J Natl Cancer Inst 2001;93(14):1054-1061.
(49) Grady WM, Carethers JM. Genomic and epigenetic instability in colorectal cancer pathogenesis. Gastroenterology 2008;135(4):1079-1099.
(50) Edmonston TB, Cuesta KH, Burkholder S et al. Colorectal carcinomas with high microsatellite instability: defining a distinct immunologic and molecular entity with respect to prognostic markers. Hum Pathol 2000;31(12):1506-1514.
(51) Soreide K, Janssen EA, Soiland H, Korner H, Baak JP. Microsatellite instability in colorectal cancer. Br J Surg 2006;93(4):395-406.
(52) Comprehensive molecular characterization of human colon and rectal cancer. Nature 2012;487(7407):330-337.