Biomarkers in colorectal cancer

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Chapter 10

General discussion and future perspectives

GENERAL DISCUSSION

During the past 2 decades, notable advances have been made in the treatment strategies of patients with colorectal cancer (CRC). For example, the introduction of total mesorectal excision (TME) combined with pre-operative (chemo)radiotherapy decreasing the local recurrence rate in rectal cancer patients dramatically [1]. In colon cancer stage III and high risk stage II, a major reduction in mortality was established by the introduction of adjuvant chemotherapy with 5-fluorouracil or capecitabine and oxaliplatin [2-4]. Until recently, adjuvant chemotherapy in rectal cancer appeared to be effective in a Japanese study, however the included patients neither received preoperative (chemo)radiotherapy nor standardized TME surgery [5]. In 2012, a Cochrane review demonstrated a beneficial effect of adjuvant chemotherapy in rectal cancer patients [6]. However, none of the 21 included trials performed TME surgery, only two trials administered pre-operative (chemo)radiotherapy and no standard definition of the rectum was used. Currently, the beneficial effect of adjuvant chemotherapy after pre-operative (chemo)radiotherapy and TME surgery has not been demonstrated, as described in chapter 2 [7-9]. Therefore, adjuvant chemotherapy in rectal cancer patients is not being used in daily clinical practice, e.g. in the Netherlands.

Treatment allocation and prognostication in patients with CRC are currently primarily influenced by the tumour, node and metastasis (TNM) classification and the circumferential resection margin [10]. Consequently, pathological staging is essential for planning the most appropriate treatment in patients with CRC. Regardless of a continuous improvement of the TNM classification, outcome among patients with the same tumour stage differ [11]. Therefore, it could be stated that the conventional classification falls short, and needs to be improved with additional biomarkers to establish well-targeted treatment strategies of individual patients. In addition, these individual treatment approaches will increase the beneficial effect of the allocated treatment and decrease adverse-events. For overview purposes this thesis was divided into three overarching parts. Part I, starts with a meta-analysis based on individual patient data. The use of adjuvant chemotherapy in patients with locally advanced rectal cancer, who underwent resection after preoperative (chemo) radiotherapy, was comprehensively evaluated. Furthermore, the use of adjuvant chemotherapy was studied at tissue level. In order to optimize the decision to offer adjuvant chemotherapy to patients with rectal cancer, histological risk factors are increasingly important. Those factors, including lymphatic invasion, perineural invasion, venous invasion and tumour budding are associated with an adverse outcome. In chapter 4, the prognostic and predictive value of these risk factors were evaluated in a rectal cancer patient cohort.
In part II the focus was on protein expression in CRC, to be more specific on proteins

involved in the evasion of immune recognition by tumour cells. This part describes the membrane expression of the classical HLA class I protein and the non-classical HLA class I protein HLA-G, in CRC. Due to the proposed immunosuppressive capabilities of the HLA-G protein, expression of this protein might participate in tumour immune surveillance. Notably because altered HLA class I expression is a well-known mechanism in escaping the anti-tumour immunity [12-15].

In part III, CRC was investigated at (epi-)genetic level. LINE-1, which constitutes approximately 17% of the human genome, was used as a marker for genome-wide hypomethylation. It has been proposed that genome-wide hypomethylation has been associated with a decreased outcome, especially in early stage colon cancer. However, large cohort studies, focussing on early stage colon cancer were lacking. Therefore, LINE-1 methylation level was investigated in a dedicated stage II colon cancer cohort. Already being used in clinic and without a doubt the single most informative genetic characteristic in early stage colon cancer is microsatellite instability (MSI). However, this established genetic marker have never been studied in a large rectal cancer cohort. Therefore, the prognostic effect of MSI has been studied in a large rectal cancer cohort in chapter 9.

PART I

ADJUVANT CHEMOTHERAPY IN LOCALLY ADVANCED RECTAL CANCER

In general, treatment of rectal cancer employs a multidisciplinary approach. Although surgery is still the cornerstone of curative treatment for non-metastasized rectal cancer. During the past 2 decades, rectal cancer treatment improved dramatically with the implementation of TME surgery. Subsequently the local recurrence rates decreased significantly [1]. The survival of rectal cancer patients is utmost determined by the development of distant metastasis, which occurs in approximately 30% [16-18]. Adjuvant chemotherapy intends to eliminate metastasizing cells in order to prevent distant recurrences. The benefit of adjuvant chemotherapy has been demonstrated in colon cancer and the advice to treat rectal cancer patients with adjuvant chemotherapy was based on extrapolation of these results. However, in rectal cancer, the beneficial effect of adjuvant chemotherapy for patients after preoperative (chemo)radiotherapy and TME surgery has not been demonstrated in randomized controlled trials, comparing adjuvant chemotherapy and observation [7-9]. Despite these results some centres in other countries still advice adjuvant chemotherapy in rectal cancer. In order to provide robust and stable evidence, a meta-analysis on individual patient data comparing adjuvant chemotherapy with observation, was performed in chapter 2. A literature search for European, randomised controlled, phase III trials, comparing adjuvant chemotherapy after preoperative (chemo)radiotherapy and surgery with observation for patients with

non-metastatic rectal cancer was performed. Four trials were eligible for inclusion and individual patient data were obtained (N=2195). In total 1196 patients were included for analysis. Overall no significant benefit of adjuvant chemotherapy compared with observed was observed in overall survival (HR 0.97, p=0.775) disease-free survival (HR 0.91, p=0.23) or cumulative incidence of distant recurrence (HR 0.61, p=0.52). Thus adjuvant chemotherapy is indeed not indicated after (chemo)radiotherapy and TME surgery in patients with locally advanced rectal cancer. To the best of our knowledge, we suppose that our meta-analysis provides currently the best available evidence on adjuvant chemotherapy in locally advanced rectal cancer.

In the meta-analysis of chapter 2, a subgroup analysis comparing the effect of adjuvant chemotherapy stratified by tumour height from the anal verge was performed. In patients with tumours located 10-15cm from the anal verge, a beneficial effect of adjuvant chemotherapy was observed, regarding disease-free survival (HR 0.59. p=0.005) and distant recurrences (HR 0.61, p=0.025). Further research for this specific subgroup of patients is essential. Therefore, we reported in chapter 3 on the results of the PROCTOR/SCRIPT trial, with a focus on patients with rectal tumours located 10-15cm from the anal verge. In the PROCTOR/SCRIPT trial, a multicentre randomized phase III trial, included patients with (y)pTNM stage II–III rectal cancer treated with preoperative (chemo)radiotherapy, were randomized to adjuvant chemotherapy or observation. In agreement with the results in the meta-analysis, a significant benefit of adjuvant chemotherapy has been observed in patients with tumours located between 10 and 15 cm from the anal verge (HR 0.58, p=0.04). Consequently, it could be discussed whether rectal tumours located 10–15 cm from the anal verge should be defined as colon cancer rather than rectal cancer, considering the favourable effect of adjuvant chemotherapy in patients with stage III and high-risk stage II colon cancer. However, in both chapters no significant interaction between distance from the anal verge and allocated treatment has been calculated.

Regarding, adequate individualized assessment, additional histological risk factors are increasingly important. Those factors, including lymphatic invasion, perineural invasion, venous invasion and tumour budding are associated with decreased outcome [19-23]. In chapter 4 the prognostic value of these above mentioned additional pathological risk factors was investigated. Furthermore, it has been proposed that these pathological markers may guide treatment decisions, regarding the use of adjuvant chemotherapy [23-25]. In chapter 4, we confirmed that stage independent pathological markers were associated with an unfavourable outcome in a dedicated rectal cancer cohort. Especially when two or more adverse prognostic pathological biomarkers were present, a strong adverse prognostic outcome has been observed. It could be

proposed that strong adverse prognostic effect observed in rectal cancer patients with ≥ 2 adverse pathological biomarkers, could be explained by the access of several routes for metastatic spread. Tumour cells can disseminate through more than one route, blood, lymph channels or along nerves, and consequently this could result in more extensive metastatic potential of the tumour. More importantly, these adverse prognostic markers did not have predictive value, and did not warrant an indication for adjuvant chemotherapy in rectal cancer treated with preoperative short course radiotherapy and TME surgery.

Besides improving oncological outcomes, the focus shifts towards reducing long-term morbidity. Accumulating evidence suggests a more important role for pre-operative chemoradiation compared with postoperative chemotherapy in rectal cancer patients [17, 26]. In addition, pre-operative chemoradiation is better tolerated compared with adjuvant treatment strategies. Nowadays, the most common used therapy is conventional long-course radiotherapy in combination with chemotherapy, followed by TME surgery. However the most optimal pre-operative radiotherapy fraction and timing of TME surgery is still under debate [27]. In a recent randomized controlled trial, comparing three pre-operative radiotherapy regimens, no significant differences have been observed regarding local recurrences, distant recurrences or overall survival [28]. Furthermore, interesting results were described in the study of Erlandsson *et al.*, by delaying surgery for 4-8 weeks after the end of short course radiotherapy, postoperative complications were significant lower, compared to short-course radiotherapy and long-course radiotherapy with delay. However, in patients with delayed surgery, 7% needed admission to the hospital due to radiation toxicity.

In conclusion, based on part I of this thesis, no significant beneficial effect of adjuvant chemotherapy after preoperative short course radiotherapy and TME surgery has been observed in a locally advanced rectal cancer. Furthermore, in patients with rectal cancer with poor prognostic histological features do not have an indication for adjuvant chemotherapy. However, the definition of the rectum remains inconsistent across counties. Based on the findings in the subgroup analysis of the performed meta-analysis in chapter 2 and chapter 3 raise the question whether rectal cancer located >10cm from the anal verge might be defined as colon tumours, considering the observed beneficial effect of adjuvant chemotherapy. However, in both chapters no significant interaction between distance from the anal verge and allocated treatment has been calculated.

PART II
TUMOUR IMMUNE INTERACTIONS, HLA-G EXPRESSION IN COLORECTAL CANCER

As originally described by Hanahan and Weinberg, the multistep process of malignant transformation acquire six biological capabilities [29]. In 2011, based on new insights emerged from the growing field of tumour-immune interactions, two additional hallmarks were added to the original six hallmarks [30]. One of these two additional markers is evasion of the immune destruction by cancer cells. Currently, the tumour immune surveillance hypothesis postulates that cancer cells are identified by the immune system and subsequently eliminated, is highly accepted.

Part II of this thesis was dedicated to the non-classical HLA-G molecule. In non-pathological conditions HLA-G is expressed at the maternal-foetal interface and immune privileges sites. *De novo* expression of HLA-G has been described in different forms of cancer and could contribute to the escape from the immune system by inhibiting NK and T cell mediated lysis [15]. Consequently HLA-G has been associated with an adverse prognosis. A second, described mechanism to escape anti-tumour immunity is to downregulate the classical HLA class I proteins. Tumour cells present tumour-associated antigens (TAA) on their cell surface, by HLA class I, following recognition and detruction by cytotoxic T-cells [9]. Previous studies reported an unfavourable prognosis in patients with cancer lacking HLA class I expression on the tumour cell surface. Moreover, an even worse survival rate has been observed when HLA class I downregulation and HLA-G expression were combined, supporting the hypothesis of an immune escape advantage for colorectal tumour cells with downregulated expression of HLA class I and *de novo* expression of HLA-G [31-33]. In order to obtain insight in the immunogenic profile of metastasizing cells, the expression of the immune-related tumour markers HLA-G and classical HLA class I in primary CRC and associated liver metastasis were investigated in chapter 5. In contrast with the proposed hypothesis, we observed that the majority of the tumour cells within the associated liver metastases did express HLA class I. Regarding HLA-G, positive IHC staining in the primary tumour was not associated with HLA-G expression in the associated liver metastasis. Therefore, HLA class I loss and *de novo* expression of HLA-G may be an advantage in escaping immune surveillance, but not mandatory for formation of metastases.

Although, IHC is a widely accepted technique, detecting the HLA-G protein with IHC remains debatable. For example, the commercially available and commonly used 4H84 mAb cross reacts with the ß2-microglubulin (ß2m) free classical HLA class I antigens on activated leucocytes [34]. This could result in false recognition of HLA-G expression in tumours that are infiltrated by leucocytes, such as CRC. Therefore, it has been recommended to use multiple HLA-G specific mAbs [34, 35]. It is crucial to note, that

the majority of the performed studies used a single mAb, most commonly 4H84. In chapter 5 three different mAbs, targeting HLA-G (4H84, MEM-G/1 and MEM-G/2) were used. Among various types of mAbs, we observed discrepant expression profiles and revealed the existence of non-specific binding. Consequently, additional biological and biochemical analysis were highly warranted to validate HLA-G expression patterns in CRC. In chapter 6, HLA-G expression was analysed in 21 recently established, low passage CRC cell lines by different methods. The DNA methylation pattern of the *HLA-G* gene and the presence of mRNA encoding HLA-G was evaluated. Membrane expression of the HLA-G protein was determined by IHC and flow cytometry. Three different anti-HLA-G mAbs were used for analysing HLA-G expression by IHC. The results obtained in the CRC cell lines were compared with paraffin-embedded tumour tissue of which the tumour cell lines were derived from. In summary, no correlation between methylation levels and mRNA expression of the *HLA-G* gene was observed. In the performed polymerase chain reaction (PCR) to detected HLA-G mRNA, a positive signal was observed in six cells lines. In four out of six of these cell lines a strong homology with isotype HLA-G3 was found after sequencing, albeit at very low levels. In correspondence with the PCR results, no HLA-G (HLA-G1) was detected with flow-cytometry. Furthermore, discrepant expression profiles were observed between the stained CRC cell lines and corresponding tumour tissue sections. Notably, discrepant expression profiles, among the used anti-HLA-G mAbs, were observed. In literature HLA-G is proposed as a promising immune check point molecule. Although, it is noticeable that the utilized methods in this research area are not selective enough to unravel all aspects of *de novo* HLA-G expression in CRC.

In chapter 7, more insight was obtained regarding discrepant expression profiles and the expression of HLA-G isoforms in CRC. Proteins of early passage CRC tumour cell lines, previously used for measuring HLA-G mRNA, were used to evaluated HLA-G expression [36]. In addition, HLA-G protein expression on fresh frozen tumour resection specimens were evaluated by western blot analysis. Furthermore, the results obtained by western blot analysis were compared with IHC on corresponding fresh frozen tissue sections. Different mAbs targeting HLA-G all HLA-G isoforms (4H84 and MEM-G/1), and mAb 5A6G7 targeting soluble HLA-G isoforms, were used to unravel the binding patterns. We showed that results obtained with IHC did not correspond with protein expression detected by western blot analysis. Furthermore, with respect to the specificity of the mAbs employed, additional immune reactivity was detected in all tumours tissues and in two out of eight CRC cell lines.

In conclusion, based on chapter 5, 6, and 7 we conclude that the role of HLA-G as immune modulator in CRC is premature. Until the time that detection of HLA-G is

selective enough to detect HLA-G expression in biological samples, rather than transfected cells or long time cultured cell lines, conclusions must be drawn with great care and therapeutic applications involving HLA-G will remain ambiguous.

PART III
PROGNOSTIC GENETIC AND EPIGENETIC BIOMARKERS IN COLORECTAL CANCER

Genome-wide DNA hypomethylation has been associated with an adverse prognosis in CRC [37-41]. It has been suggested that a decrease in global DNA methylation is related to hypomethylated LINE-1 elements, therefore LINE-1 methylation serves as a surrogate marker for overall DNA methylation status [42]. In chapter 8, the prognostic value of LINE-1 methylation was studied in a stage II colon cancer cohort of 164 patients. Stage II colon cancer patients with decreased LINE-1 methylation levels had a significantly unfavourable overall survival compared with patients with a higher level of tumour DNA methylation. In patients aged over 65 years, this effect was more prominent, supporting the role of LINE-1 methylation level as prognostic biomarker. On the other hand, the observed difference in overall survival were not reflected in the disease-free survival (DFS) or relapse free survival (RFS). Consequently, a specific role for LINE-1 DNA methylation level as a prognostic biomarker for disease progression could not be confirmed. In literature, multiple studies did not observe a correlation between LINE-1 hypomethylation and survival in more advanced disease stages [38, 41, 43]. Consequently, global loss of DNA methylation appears to be more an early event in colon cancer formation rather than a contributor to disease progression. This hypothesis is supported by the result published by Pavicic *et al*., comparing LINE-1 methylation levels in normal mucosa of CRC patient with sporadic CRC, familiar CRC and in patients with hereditary nonpolyposis colorectal cancer (HNPCC) [44]. The lowest LINE-1 methylation levels were observed in normal mucosa of patients with familial CRC, indicating that lower levels of LINE-1 methylation predispose normal colon tissue to cancer development. Furthermore, studies report that normal colon mucosa harbours an age-related global hypomethylation [45, 46]. This suggests that a decrease in DNA methylation is a contributing factor to the rising incidence of CRC in older aged people. Furthermore, models have been developed to predict chronological age from the level of DNA methylation [47, 48]. It has been suggested that patients with a discrepant result between chronological age and age predicted based on DNA methylation patterns do have an increased mortality risk. For example, age predicted based on DNA methylation exceeding chronological age could be associated with an unfavourable survival.

Thus global hypomethylation might function as a “hit” in Knudsons “multi-hit” hypothesis, supporting the hypothesis of global hypomethylation as “driver” in carcinogenesis instead of a prognostic factor for disease progression in colon cancer

patients. In other words, it could be advocated that global LINE-1 hypomethylation appears to be an early event in colon cancer formation.

A well-known genetic contributor to CRC formation is MSI and has been associated with a prognostic advantage in early stage rectal cancer [49-51]. Therefore, routine screening for MSI in patients with newly diagnosed CRC has been supported by the guidelines from American Society of Clinical oncology (ASCO) and the European Society for Medical Oncology (ESMO) [52, 53]. In contrast to colon cancer, the implications of rectal cancer with MSI remain undefined, though highly relevant to enable the development of treatment strategies driven by biomarkers. Therefore, we determined the role of MSI in respect to prevalence and outcome in patients with rectal cancer who participated in two prospective phase III trials (TME trial and PROCTOR-SCRIPT trial). Due to the relative low incidence of MSI in rectal cancer, limited evidence regarding the prognostic and predictive value of MSI in rectal cancer exists. To the best of our knowledge, we studied the prognostic value of MSI in the largest rectal cancer cohort, currently available. In line with the literature, MSI was present in 3.8% of the patients. Furthermore, no effect of MSI was observed regarding overall survival, distant recurrence and local recurrence. As shown in chapter 9, studies reporting on MSI as a prognostic factor in rectal cancer patients were conflicting. For example, Colombino *et al.,* showed significant better overall survival en disease-free survival for patients with MSI tumours [54]. In contrast, significant worse survival rates for patients with MSI rectal cancer have been reported. Samowitz *et al.* [55]. In addition, a comparison of the currently available literature has been difficult because the standard treatment regimens for rectal cancer have been changed over time. Therefore, a paucity of evidence exists regarding long-term survival data in MSI rectal cancer patients treated with conventional therapy. However, to the best of our knowledge, our study has the largest rectal cancer cohort with known treatment strategies. In conclusion, rectal cancer patients with MSI is a distinct subclass of CRC, however, given the relative low incidence of MSI in rectal cancer it will be challenging to complete unravel the influence of MSI on prognosis.

FUTURE DIRECTIONS

The TNM classification alone does not have the potential to provide most optimal and adequate individualized assessment. Therefore conventional staging needs to be supplemented with additional biomarkers to improve personalized treatment allocation in CRC. Despite, intensive research on genomic, epigenetic, molecular and clinicopathological data the use of biomarkers in daily practice falls short. During the process of tumour development, tumour cells may acquire multiple capabilities, therefore multiple biomarkers should be combined for prognostic and predictive purposes, rather than the use of one single biomarker. Moreover, biomarker studies would be more valuable when performed on pre-treatment tumour biopsies, since the most important application of a biomarker will be to guide treatment strategies. Especially for rectal cancer, information from biopsies might influence timing and type of preoperative treatment allocated in rectal cancer. Currently, preoperative (chemo) radiotherapy and TME surgery has improved local control, although no effect on overall survival has been demonstrated. Moreover, as demonstrated in chapter 1 adjuvant chemotherapy did not improve survival after (chemo)radiotherapy and TME surgery. An alternative for eliminating possible micometastatic cells could be the administration of preoperative systemic chemotherapy. This shift towards more intensive pre-operative treatment is currently ongoing and the results from the RAPIDO trial are awaited. Presumably pre-operative chemotherapy could reach a higher dose intensity and compliance then postoperative chemotherapy. Furthermore, it has been shown that intensive pre-operative treatment strategies could induce a pathological complete response in approximately 30% of the patients. Subsequently, the key question is; could major surgery be avoided in these rectal cancer patients? The so-called "watch-and-wait" approach has emerged as a treatment strategy for rectal cancer, as introduced by Harb-Gama and recently described by van der Valk *et al.* [56,57]. The ultimate challenge for the upcoming years, will be to further extend the knowledge regarding predicting treatment response. In order to achieve precision medicine, specialists involved in CRC management need to collaborate to provide the most effective and tolerated treatment as ultimate goal.

REFERENCES

1. Kapiteijn E, Marijnen CA, Nagtegaal ID et al. Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer. N Engl J Med 2001; 345: 638-646.
2. Andre T, Boni C, Mounedji-Boudiaf L et al. Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. N Engl J Med 2004; 350: 2343-2351.
3. 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: 3109-3116.
4. Andre T, Sargent D, Tabernero J et al. Current issues in adjuvant treatment of stage II colon cancer. Ann Surg Oncol 2006; 13: 887-898.
5. Akasu T, Moriya Y, Ohashi Y et al. Adjuvant chemotherapy with uracil-tegafur for pathological stage III rectal cancer after mesorectal excision with selective lateral pelvic lymphadenectomy: a multicenter randomized controlled trial. Jpn J Clin Oncol 2006; 36: 237-244.
6. Petersen SH, Harling H, Kirkeby LT et al. Postoperative adjuvant chemotherapy in rectal cancer operated for cure. Cochrane Database Syst Rev 2012; CD004078.
7. Bosset JF, Calais G, Mineur L et al. Fluorouracil-based adjuvant chemotherapy after preoperative chemoradiotherapy in rectal cancer: long-term results of the EORTC 22921 randomised study. Lancet Oncol 2014; 15: 184-190.
8. Breugom AJ, van Gijn W, Muller EW et al. Adjuvant chemotherapy for rectal cancer patients treated with preoperative (chemo)radiotherapy and total mesorectal excision: a Dutch Colorectal Cancer Group (DCCG) randomized phase III trial. Ann Oncol 2015; 26: 696-701.
9. Glynne-Jones R, Counsell N, Quirke P et al. Chronicle: results of a randomised phase III trial in locally advanced rectal cancer after neoadjuvant chemoradiation randomising postoperative adjuvant capecitabine plus oxaliplatin (XELOX) versus control. Ann Oncol 2014; 25: 1356-1362.
10. Sobin L H GMK, Wittekind Ch. TNM classification of malignant tumours Chichester, West Sussex, UK ; Hoboken, NJ : Wiley-Blackwell, 2010.
11. Puppa G, Sonzogni A, Colombari R, Pelosi G. TNM staging system of colorectal carcinoma: a critical appraisal of challenging issues. Arch Pathol Lab Med 2010; 134: 837-852.
12. Algarra I, Garcia-Lora A, Cabrera T et al. The selection of tumor variants with altered expression of classical and nonclassical MHC class I molecules: implications for tumor immune escape. Cancer Immunol Immunother 2004; 53: 904-910.
13. Atkins D, Breuckmann A, Schmahl GE et al. MHC class I antigen processing pathway defects, ras mutations and disease stage in colorectal carcinoma. Int J Cancer 2004; 109: 265-273.
14. Momburg F, Degener T, Bacchus E et al. Loss of HLA-A,B,C and de novo expression of HLA-D in colorectal cancer. Int. J. Cancer 1986; 37: 179-184.
15. Wischhusen J, Waschbisch A, Wiendl H. Immune-refractory cancers and their little helpers--an extended role for immunetolerogenic MHC molecules HLA-G and HLA-E? Semin Cancer Biol 2007; 17: 459-468.
16. Engelen SM, Maas M, Lahaye MJ et al. Modern multidisciplinary treatment of rectal cancer based on staging with magnetic resonance imaging leads to excellent local control, but distant control remains a

challenge. Eur J Cancer 2013; 49: 2311-2320.

17. Sauer R, Liersch T, Merkel S et al. Preoperative versus postoperative chemoradiotherapy for locally advanced rectal cancer: results of the German CAO/ARO/AIO-94 randomized phase III trial after a median follow-up of 11 years. J Clin Oncol 2012; 30: 1926-1933.
18. van Gijn W, Marijnen CA, Nagtegaal ID et al. Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer: 12-year follow-up of the multicentre, randomised controlled TME trial. Lancet Oncol 2011; 12: 575-582.
19. Betge J, Pollheimer MJ, Lindtner RA et al. Intramural and extramural vascular invasion in colorectal cancer: prognostic significance and quality of pathology reporting. Cancer 2012; 118: 628-638.
20. Chand M, Siddiqui MR, Swift I, Brown G. Systematic review of prognostic importance of extramural venous invasion in rectal cancer. World J Gastroenterol 2016; 22: 1721-1726.
21. Choi HJ, Park KJ, Shin JS et al. Tumor budding as a prognostic marker in stage-III rectal carcinoma. Int J Colorectal Dis 2007; 22: 863-868.
22. Knijn N, Mogk SC, Teerenstra S et al. Perineural Invasion is a Strong Prognostic Factor in Colorectal Cancer: A Systematic Review. Am J Surg Pathol 2016; 40: 103-112.
23. Nikberg M, Chabok A, Letocha H et al. Lymphovascular and perineural invasion in stage II rectal cancer: a report from the Swedish colorectal cancer registry. Acta Oncol 2016; 55: 1418-1424.
24. Quah HM, Chou JF, Gonen M et al. Identification of patients with high-risk stage II colon cancer for adjuvant therapy. Dis Colon Rectum 2008; 51: 503-507.
25. Petrelli F, Pezzica E, Cabiddu M et al. Tumour Budding and Survival in Stage II Colorectal Cancer: a Systematic Review and Pooled Analysis. J Gastrointest Cancer 2015; 46: 212-218.
26. Park JH, Yoon SM, Yu CS et al. Randomized phase 3 trial comparing preoperative and postoperative chemoradiotherapy with capecitabine for locally advanced rectal cancer. Cancer 2011; 117: 3703-3712.
27. Glimelius B. Optimal Time Intervals between Pre-Operative Radiotherapy or Chemoradiotherapy and Surgery in Rectal Cancer? Front Oncol 2014; 4: 50.
28. Erlandsson J, Holm T, Pettersson D et al. Optimal fractionation of preoperative radiotherapy and timing to surgery for rectal cancer (Stockholm III): a multicentre, randomised, non-blinded, phase 3, non-inferiority trial. Lancet Oncol 2017; 18: 336-346.
29. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000; 100: 57-70.
30. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011; 144: 646-674.
31. Reimers MS, Engels CC, Putter H et al. Prognostic value of HLA class I, HLA-E, HLA-G and Tregs in rectal cancer: a retrospective cohort study. BMC Cancer 2014; 14: 486.
32. Watson NF, Ramage JM, Madjd Z et al. Immunosurveillance is active in colorectal cancer as downregulation but not complete loss of MHC class I expression correlates with a poor prognosis. Int. J. Cancer 2006; 118: 6-10.
33. Zeestraten EC, Reimers MS, Saadatmand S et al. Combined analysis of HLA class I, HLA-E and HLA-G predicts prognosis in colon cancer patients. Br. J. Cancer 2014; 110: 459-468.
34. Polakova K, Kuba D, Russ G. The 4H84 monoclonal antibody detecting beta2m free nonclassical HLA-G molecules also binds to free heavy chains of classical HLA class I antigens present on activated

lymphocytes. Hum Immunol 2004; 65: 157-162.
35. Apps R, Gardner L, Moffett A. A critical look at HLA-G. Trends Immunol 2008; 29: 313-321.
36. Swets M, Seneby L, Boot A et al. Promoter methylation and mRNA expression of HLA-G in relation to HLA-G protein expression in colorectal cancer. Hum Immunol 2016; 77: 764-772.
37. Baba Y, Huttenhower C, Nosho K et al. Epigenomic diversity of colorectal cancer indicated by LINE-1 methylation in a database of 869 tumors. Mol Cancer 2010; 9: 125.
38. Benard A, van de Velde CJ, Lessard L et al. Epigenetic status of LINE-1 predicts clinical outcome in early-stage rectal cancer. Br J Cancer 2013; 109: 3073-3083.
39. Mima K, Nowak JA, Qian ZR et al. Tumor LINE-1 methylation level and colorectal cancer location in relation to patient survival. Oncotarget 2016.
40. Ogino S, Nosho K, Kirkner GJ et al. A cohort study of tumoral LINE-1 hypomethylation and prognosis in colon cancer. J Natl Cancer Inst 2008; 100: 1734-1738.
41. Sunami E, de Maat M, Vu A et al. LINE-1 hypomethylation during primary colon cancer progression. PLoS One 2011; 6: e18884.
42. Yang AS, Estecio MR, Doshi K et al. A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. Nucleic Acids Res 2004; 32: e38.
43. Murata A, Baba Y, Watanabe M et al. Methylation levels of LINE-1 in primary lesion and matched metastatic lesions of colorectal cancer. Br J Cancer 2013; 109: 408-415.
44. Pavicic W, Joensuu EI, Nieminen T, Peltomaki P. LINE-1 hypomethylation in familial and sporadic cancer. J Mol Med (Berl) 2012; 90: 827-835.
45. Ahuja N, Issa JP. Aging, methylation and cancer. Histol Histopathol 2000; 15: 835-842.
46. Suzuki K, Suzuki I, Leodolter A et al. Global DNA demethylation in gastrointestinal cancer is age dependent and precedes genomic damage. Cancer Cell 2006; 9: 199-207.
47. Lin Q, Weidner CI, Costa IG et al. DNA methylation levels at individual age-associated CpG sites can be indicative for life expectancy. Aging (Albany NY) 2016; 8: 394-401.
48. Marioni RE, Shah S, McRae AF et al. DNA methylation age of blood predicts all-cause mortality in later life. Genome Biol 2015; 16: 25.
49. Guastadisegni C, Colafranceschi M, Ottini L, Dogliotti E. Microsatellite instability as a marker of prognosis and response to therapy: a meta-analysis of colorectal cancer survival data. Eur J Cancer 2010; 46: 2788-2798.
50. Hutchins G, Southward K, Handley K et al. Value of mismatch repair, KRAS, and BRAF mutations in predicting recurrence and benefits from chemotherapy in colorectal cancer. J Clin Oncol 2011; 29: 1261-1270.
51. Sargent DJ, Marsoni S, Monges G et al. Defective mismatch repair as a predictive marker for lack of efficacy of fluorouracil-based adjuvant therapy in colon cancer. J Clin Oncol 2010; 28: 3219-3226.
52. Balmana J, Balaguer F, Cervantes A et al. Familial risk-colorectal cancer: ESMO Clinical Practice Guidelines. Ann Oncol 2013; 24 Suppl 6: vi73-80.
53. Stoffel EM, Mangu PB, Gruber SB et al. Hereditary colorectal cancer syndromes: American Society of Clinical Oncology Clinical Practice Guideline endorsement of the familial risk-colorectal cancer: European Society for Medical Oncology Clinical Practice Guidelines. J Clin Oncol 2015; 33: 209-217.

54. Colombino M, Cossu A, Manca A et al. Prevalence and prognostic role of microsatellite instability in patients with rectal carcinoma. Ann Oncol 2002; 13: 1447-1453.
55. Samowitz WS, Curtin K, Wolff RK et al. Microsatellite instability and survival in rectal cancer. Cancer Causes Control 2009; 20: 1763-1768.
56. Habr-Gama A, de Souza PM, Ribeiro U, Jr. et al. Low rectal cancer: impact of radiation and chemotherapy on surgical treatment. Dis Colon Rectum 1998; 41: 1087-1096.
57. van der Valk MJM, Hilling DE, Bastiaannet E et al. Long-term outcomes of clinical complete responders after neoadjuvant treatment for rectal cancer in the International Watch & Wait Database (IWWD): an international multicentre registry study. Lancet 2018; 391: 2537-2545.