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**Biomarkers in colorectal cancer**

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# Biomarkers in colorectal cancer

Marloes Swets

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# Biomarkers in colorectal cancer

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# Chapter 1

## General introduction and thesis outline

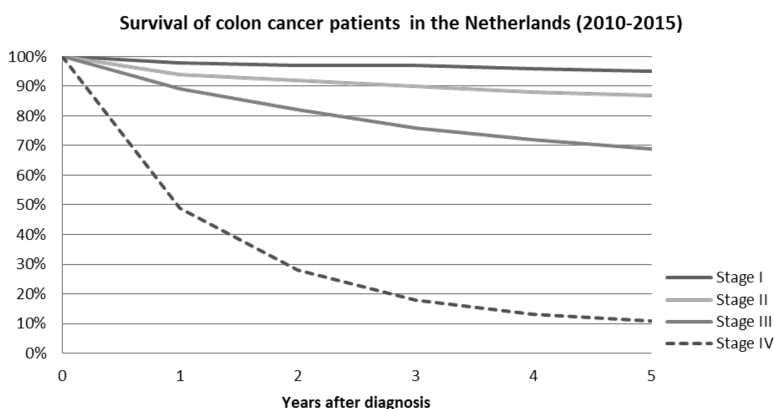
## COLORECTAL CANCER INCIDENCE, TREATMENT AND PROGNOSIS

Colorectal cancer (CRC) is the second most common diagnosed cancer in females and the third most common cancer in males and accounts for the second cause of cancer death in Europe with an estimated incidence of 447.000 new CRC cases and 215.000 deaths, in 2012 [1]. As shown in figure 1, the estimation of the prognosis is mainly determined by conventional staging such as, tumour, node and metastasis (TNM) classification. Despite a continuous improvement of the TNM classification, outcome among patients with the same tumour stage varies significantly [2]. Consequently, it could be stated that adequate individualized assessment could not be accomplished with conventional classification. Furthermore, evidence is accumulating that rectal tumours differ from colon tumours [3,4], resulting in a separation of colon and rectal cancer regarding biology and treatment.

In general, treatment of CRC employs a multidisciplinary approach, though surgery remains the cornerstone of curative treatment for non-metastasized CRC in most cases. Prior to surgery, clinical staging is performed by a combination of endoscopy, CT (Computerized Tomography) and in rectal cancer also MRI (Magnetic Resonance Imaging). For colon cancer stage III and high risk stage II, surgery is followed by adjuvant chemotherapy [5-7]. Still, approximately 20% of the patients with stage I-III colon cancer, develop metastatic disease within 5 years [8]. For rectal cancer, important advances have been made in treatment with the implementation of total mesorectal excision (TME) [9], combined with preoperative (chemo)radiotherapy and the ability to more accurately stage rectal cancer with MRI. However, approximately 30% of the patients with rectal cancer treated with a curative intent will develop distant metastasis [10-12]. Adjuvant chemotherapy was thought to prevent distant metastasis by eliminating micrometastases and circulating tumour cells. However, the advantageous effect of adjuvant chemotherapy for patients with stage II/III rectal cancer, treated with preoperative (chemo)radiotherapy and TME surgery, is not accepted as a standard in according to ESMO guidelines [13]. In the PROCTOR-SCRIPT trial, a multicentre randomized phase III trial, patients were randomized between adjuvant chemotherapy or observation in patients with (y)pTNM stage II-III rectal cancer treated with preoperative (chemo)radiotherapy and TME surgery. This study showed no survival benefit for patients with (y)pTNM stage II-III rectal cancer treated with adjuvant chemotherapy compared to observation [14]. In order to provide robust and stable evidence for the use of adjuvant chemotherapy in patients with locally advanced rectal cancer, a meta-analysis based on individual patient data was performed in this thesis. Furthermore, accumulating evidence suggests a more important role for preoperative chemo radiation therapy (CRT) compared with postoperative CRT in rectal cancer patients [11,15]. Trials with intensified preoperative treatment, such as the RAPIDO trial, are in progress, and results

are awaited [16]. Furthermore, in patients with locally advanced rectal cancer receiving preoperative chemo radiation a complete pathological response has been observed in 20-30%, consequently there is an emerging role for watch-and-wait strategies as introduced by Habr-Gama [17]. However, more data and long term outcomes are needed before this organ-preservation strategy could be incorporated safely. Therefore, The European Registration of Cancer Care, and the Cahmpalimaud foundation has initiated the International Watch and Wait Database to collected uniform data [18].

Currently, in contrast to colon cancer for rectal cancer, there are no molecular markers for rectal cancer that evaluate, whether a patient benefit from preoperative treatment, predict response to (chemo)radiotherapy or whether a tumour will metastasize.



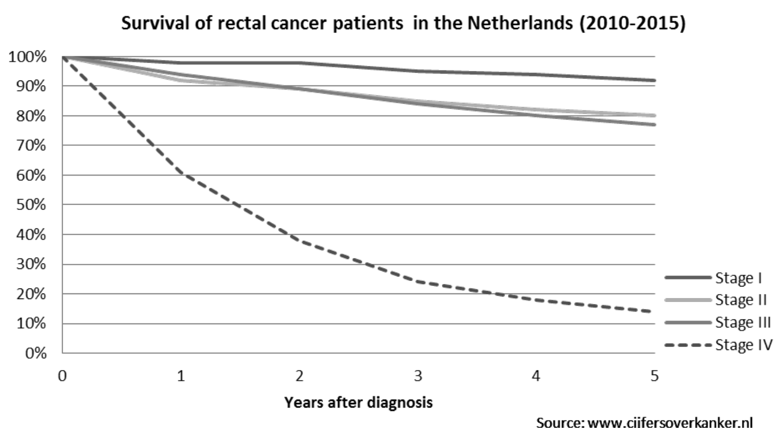




**Figure 1:** Survival curves. Upper panel shows survival of colon cancer patients stratified by disease stage. Lower panel shows survival of rectal cancer patients stratified by disease stage.

### PROGNOSTIC AND PREDICTIVE BIOMARKERS IN CRC

Treatment choices are mainly influenced by the TNM classification, which provides an estimation of the clinical prognosis and creates uniformity in the diagnosis of oncologic diseases [19,20]. Although, outcome among patients with the same tumour stage differs [2]. Insight into the biological diversity of CRCs in relation to clinical features is needed, to ultimately find the right equilibrium in the treatment to avoid mortality and morbidity and prevent over- and under treatment.

Biomarkers are biological entities that can be measured, for example in blood or tumour tissue, to be used as indicators of pathological processes. Investigating biomarkers that reflect tumour growth and metastatic potential can provide information on the clinical outcome, based on the underlying biological mechanism. The detection of prognostic and predictive biomarkers has become a crucial part of CRC research. Despite encouraging preliminary data, so far the use of biomarkers in clinical practice is very limited. In CRC, only a few biomarkers are used in daily clinical practice, such as *RAS/RAF* and microsatellite status. For example, it has been demonstrated that *RAS* mutations were found in patients who were resistant to monoclonal antibodies targeting the epidermal growth factor receptor [21]. A second well known example is microsatellite instability (MSI), which is without doubt the single most informative genetic characteristic in early stage colon cancer. In contrast to colon cancer, the implications of a MSI tumour located in the rectum remain undefined.  Besides the fact that MSI is a hallmark of hereditary non-polyposis colorectal cancer (HNPCC), MSI is found in approximately 15% of the sporadic CRC tumours [22]. In addition, accumulating evidence advocates that deficient mismatch repair mechanism, especially in early-stage colon cancer, is associated with a clinical prognostic advantage [23-25], in comparison with microsatellite stable (MSS) colon tumours. In contrast, an adverse prognostic effect of MSI was observed in metastatic CRC [26]. On the predictive value of MSI regarding the response to 5-fluorouracil (5-FU), although with conflicting results, [25,27-29], accumulating preclinical and clinical evidence reports a resistance to 5-fluorouracil (5-FU), in patients with deficient MMR tumours [24,25,30,31].

## SOURCES OF BIOMARKERS IN CRC

### *Tumour-immune interactions*

Molecular mechanisms responsible for tumour genesis are likely to influence clinical outcome. In 2000, it has been proposed that six biological alterations must be acquired during the multistep development of cancer [32]. These well-known six hallmarks of cancer consist of: sustaining proliferative signalling, activating tissue invasion, evading growth

suppression and metastasis, enabling replicative immortality, inducing angiogenesis, and resisting apoptosis. After the recognition of the importance of tumour microenvironment, additional hallmarks were added in 2011 of which evasion of the immune recognition was one of these emerging hallmarks [33]. The concept of tumour immune-editing, in order to escape the host defence immunity is currently widely accepted [34]. A well described mechanism, in the escape from the host immune recognition and destruction is complete loss or downregulation of classical HLA class I molecules. Downregulation or complete loss of HLA class I diminishes tumour-associated antigen presentation on the cell membrane. Consequently, cytotoxic T-cells recognition and destruction of tumour cells is minimized [35,36]. Another mechanism in escaping the anti-tumour immune response, could be *de novo* expression of non-classical HLA class I proteins on the cell surface, such as HLA-G a molecule with important immunomodulatory properties. HLA-G is rarely expressed in non-pathological conditions, other than in immune privileged sites, such as placenta tissue, where it is involved in fetal immune tolerance towards the maternal immune system [37,38]. Alternative splicing of the primary HLA-G transcript has been described in literature, resulting in seven HLA-G isoforms: four membrane bound (HLA-G1, G2, G3 and G4) and three soluble isoforms (HLA-G5, G6 and G7) [39]. Furthermore, HLA-G expression in a *de novo* matter has been reported in human tumour cells, including CRC [40-42]. The influence of the tumour-driven *de novo* expression of HLA-G in escaping immune surveillance is by interaction with inhibitory receptors on T lymphocytes and natural killer (NK) cells [40], in other words HLA-G functions as an immune checkpoint inhibiting antitumor responses. This could explain why expression of the HLA-G protein on tumour cells might be associated with higher tumour grade and adverse prognosis [43]. Therefore, HLA-G has been proposed as a potential target for immunotherapy strategies [44]. However, it should be noted that discrepancies among and within different tumour types were reported. For, CRC, HLA-G expression, detected with immunohistochemistry (IHC), varies from 20-72% [44,45]. IHC is a widely accepted technique, although remains controversial in detecting HLA-G protein expression [46,47]. To firmly evaluate HLA-G protein expression additional molecular and biochemical analysis are essential. Thereby, HLA-G protein expression should be investigated in patient derived samples, rather than (cancer) cell lines. In this thesis, HLA-G expression was intensively investigated in both CRC cell lines and patient derived samples. Moreover, different biochemical techniques to detect HLA-G were used and results will be compared in order to firmly evaluate whether or not results obtained by IHC will be reliable and if HLA-G indeed plays an important role in CRC.

***Tumour genetics and epigenetics***

Currently, the first genetic biomarkers are used clinically, such as *RAS/RAF* and MSI status. In the current guidelines it has been recommended to determine *RAS* and *BRAF*

mutation status in patients with irresistible CRC metastasis . Since patient with *BRAF* mutated tumours do not benefit from anti-epidermal growth factor receptor (EGFR) treatment [48]. In addition, in patients with wild-type *RAS/BRAF* metastasized colon cancer, anti-EGFR therapy is only recommended in colon cancer patients with left-sided primary tumours [49,50].

As earlier mentioned, approximately 15% of the sporadic stage II-III CRC has MSI [22] and in addition MSI tumours have distinct features such as a more proximal localization, higher grade, a mucinous histology with tumour infiltrating lymphocytes and the presence of a *BRAF* mutation. Furthermore, a prognostic advantage has been observed for MSI tumours [23-25]. Currently, accumulating evidence illustrates the significance of determining MSI in colon cancer. Besides a prognostic effect, MSI status is predictive of response to adjuvant chemotherapy. For example, in patients with high-risk stage II colon cancer with MSI tumours, no beneficial effect of adjuvant chemotherapy has been observed, indicating that these patients should not treated with 5-FU-based adjuvant chemotherapy [25,51,52]. Therefore, the routine screening for deficient mismatch repair (MMR) mechanisms in patients with newly diagnosed CRC has been supported by the guidelines from American Society of Clinical oncology (ASCO), the European Society for Medical Oncology (ESMO) and has been implemented in the most recent edition of the TNM staging classification (eight edition, 2017) [53-55]. In contrast to colon cancer, the role of a MSI in rectal cancer remain undefined. The long-term prognosis of MSI in sporadic rectal cancers has not been well-established in large patient cohorts, although it may be highly relevant to enable the implementation of personalized treatment strategies driven by biomarkers. Furthermore, an increased radio-sensitivity in MSI tumours has been suggested based on *in vitro* experiments and in small patient series [56,57]. Accordingly, the clinical significance of MSI in rectal cancer needs to be evaluated in a large rectal cancer cohort.

Interestingly, MMR germline mutations are found in patients with HNPCC, although in sporadic CRC MSI results most frequently from inactivation of the *MLH1* gene by hypermethylation of CpG islands in the promoter region [58,59]. This example illustrates the important role of epigenetics in carcinogenesis. Epigenetics has become a recent focus in cancer research. Besides hypermethylation, genome-wide DNA hypomethylation is an crucial epigenetic alteration in cancer too. In CRC, hypomethylation is considered as an early event in the carcinogenesis and thereby contributing to genomic instability [60,61]. To indirectly measure global hypomethylation, the methylation status of long interspersed nucleotide element (LINE-1) repeats can be used as surrogate marker [62]. LINE-1 repeats make-up approximately 17% of the human genome and are present on most of the chromosomes [63]. Tumour LINE-1 methylation status is intensively

studied and a decrease of LINE-1 methylation has been observed in almost all human malignancies [64,65]. LINE-1 hypomethylation in CRC is thought to be associated with an adverse prognosis, which suggests a role for LINE-1 as prognostic biomarker [66]. In the current available literature tumour LINE-1 methylation status has been related to clinical outcome in CRC. However, LINE-1 methylation status was predominantly investigated in study cohorts consisting of rectal and colon cancer patients together. Compelling evidence illustrates that rectal cancers biologically differ significantly from colon cancer [3,4]. For rectal cancer it has been demonstrated by Benard *et al.* that LINE-1 hypomethylation was associated with an unfavourable survival and higher tumour recurrence rates, in early stage [67]. Large patient studies on exclusively patients with early stage colon cancer are not available in the current literature. Therefore, studies investigating the prognostic role of tumour LINE-1 methylation level in stage II colon cancer specifically are needed. In this thesis we aimed to investigate the prognostic role of tumour LINE-1 methylation level in stage II colon cancer, in order to identify high-risk patient to ultimately avoid over-, or under treatment.

## OUTLINE OF THE THESIS

Potential relevant biomarkers can be found at different levels in tumour development and disease progression. This thesis is divided into three overarching parts. Colorectal cancer was studied from a population-based perspective (part I) to a molecular level, detailed as protein expression (part II) and (epi)genetics (part III), as indicated in Figure 2. In part I the use of adjuvant chemotherapy in patients with locally advanced rectal cancer, who underwent resection after preoperative (chemo)radiotherapy, was evaluated in a meta-analysis based on individual patient data. Since four randomized controlled trials individually did not end the ongoing debate about the role of adjuvant chemotherapy [14,68-70]. In part II the ability by tumour cells to evade the immune recognition was studied, especially the role of the non-classical HLA class I molecule HLA-G was studied in detail. In part III, an epigenetic biomarker, LINE-1 methylation level, was studied in a dedicated stage II colon cohort. In addition, an established genetic biomarker for colon cancer, MSI, was studied in a large rectal cancer cohort.

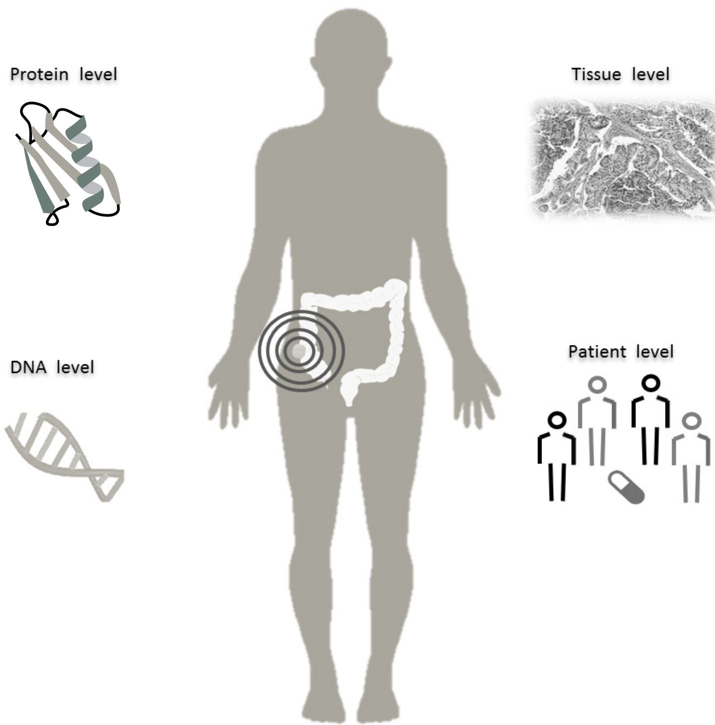



**Figure 2:** Global overview of different levels involved in colorectal cancer formation and disease progression investigated in this thesis.

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# PART I

## Adjuvant chemotherapy in rectal cancer treatment

# Chapter 2

## Adjuvant chemotherapy after preoperative (chemo)radiotherapy and surgery for patients with rectal cancer: a systematic review and meta-analysis of individual patient data

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# ABSTRACT

## BACKGROUND

The role of adjuvant chemotherapy for patients with rectal cancer after preoperative (chemo)radiotherapy and surgery is uncertain. We performed an individual patient data meta-analysis to compare adjuvant chemotherapy with observation in patients with rectal cancer.

## METHODS

We searched PubMed, MEDLINE, Embase, Web of Science, The Cochrane Library, CENTRAL, and conference abstracts to identify published and unpublished European randomised, controlled, phase III trials comparing observation with adjuvant chemotherapy after preoperative (chemo)radiotherapy and surgery for patients with non-metastatic rectal cancer. Primary end-point was overall survival. Secondary end-points were disease-free survival and distant recurrence rate.

The hazard ratios (HRs) and 95% confidence intervals (CIs) for overall survival, disease-free survival, and cumulative incidence of distant recurrences were calculated with Cox proportional hazards model. The regression models included strata defined by a term representing the distinct trials.

## FINDINGS

We included 1196 patients for analyses. For sensitivity analysis (all patients from eligible trials), 2195 patients were included. No significant differences in overall survival were found (HR 0·97, 95% CI 0·81-1·17, p=0·775) between the observation and chemotherapy arm. There were also no significant differences in overall survival for subgroups. Sensitivity analysis showed a HR of 0·95 (95% CI 0·82-1·09, p=0·430) for overall survival. Overall, no benefit of adjuvant chemotherapy was demonstrated for disease-free survival (HR 0·91, 95% CI 0·77-1·07, p=0·230) and distant recurrences (HR 0·94, 95% CI 0·78-1·14, p=0·523).

In subgroup analysis, patients with a tumour between 10 cm and 15 cm from the anal verge who received adjuvant chemotherapy had an improved disease-free survival (HR 0·59, 95% CI 0·40-0·85, p=0·005, $p_{interaction}$=0·107) and distant recurrence rate (HR 0·61, 95% CI 0·40-0·94, p=0·025, $p_{interaction}$=0·126).

## INTERPRETATION

Overall, 5-FU based adjuvant chemotherapy did not improve overall survival, disease-free survival and distant recurrence rate. However, our findings suggest that patients with a tumour located between 10 cm and 15 cm from the anal verge may benefit

from adjuvant chemotherapy in terms of disease-free survival and distant recurrences. Further research with regard to preoperative and postoperative treatment for this subgroup of patients is warranted.



# INTRODUCTION

Important advances have been made in rectal cancer treatment with the introduction of total mesorectal excision (TME), the addition of preoperative (chemo)radiotherapy to TME, and the ability of more accurate staging with magnetic resonance imaging (MRI).[1-9] Although locoregional recurrence rates and survival improved over the past years, distant recurrence rates did not. Unfortunately, still about 30% of all patients treated with curative intent will eventually develop distant metastases.[3, 6, 9] Adjuvant chemotherapy might decrease distant metastases by eliminating circulating tumour cells and micrometastases. However, the use of adjuvant chemotherapy in rectal cancer patients treated with preoperative (chemo)radiotherapy and surgery is still under debate.[10] For patients treated without preoperative (chemo)radiotherapy and TME surgery which results in high locoregional recurrence rates, adjuvant chemotherapy showed to be effective. This is demonstrated in a Cochrane review by Petersen et al. showing a risk reduction of 17% (HR 0·83, 95% CI 0·76-0·91) on overall survival and 25% (HR 0·75, 95% CI 0·68-0·83) on disease-free survival for patients who received adjuvant chemotherapy.[11] In this Cochrane review, only two studies administered preoperative (chemo)radiotherapy[12, 13] Of these, the EORTC 22921 study[12] did not demonstrate a benefit of adjuvant chemotherapy, while the QUASAR[13] did show a borderline significant improvement in overall survival for patients with rectal cancer. However, in the QUASAR study, only 21% of patients with rectal cancer or both colon and rectal cancer received preoperative radiotherapy.[13] Furthermore, a Japanese trial also demonstrated an improved overall and disease-free survival for stage III rectal cancer patients who were randomised to adjuvant chemotherapy after standardised mesorectal excision.[14] However, none of the patients received preoperative (chemo)radiotherapy and standardised mesorectal excision included selective lateral lymphadenectomy.[14]

In contrast, more recent trials comparing adjuvant chemotherapy and observation after preoperative (chemo)radiotherapy and TME surgery all did not demonstrate a benefit of adjuvant chemotherapy.[7, 15-17] With this individual patient data meta-analysis, we aim to investigate the effect of adjuvant 5-fluorouracil/leucovorin (5-FU/LV) based chemotherapy compared with observation after preoperative (chemo)radiotherapy and surgery for rectal cancer patients.

# METHODS



## SEARCH STRATEGY AND SELECTION CRITERIA

In cooperation with a trained librarian, we performed a search to identify published and unpublished European randomised, controlled, phase III trials comparing observation with adjuvant chemotherapy after preoperative (chemo)radiotherapy and surgery for patients with non-metastatic rectal cancer. Patients aged 18 years and older were eligible for inclusion. All current available preoperative treatment regimens, as well as both total mesorectal excision (TME) and conventional surgery were accepted for inclusion. Randomised controlled trials on adjuvant chemotherapy without an observation arm were excluded.

We searched PubMed, MEDLINE (OVID version), Embase (OVID version), Web of Science, The Cochrane Library, and CENTRAL from the date of their inception until June 26$^{th}$, 2014 for relevant articles. We also searched abstracts from the most important international meetings. The search strategy consisted of the "AND" combination of three main concepts: "rectal carcinoma", "adjuvant chemotherapy", and "preoperative treatment". All relevant keyword variations were used for these three main concepts. Searches were limited to reports published in English. Literature screening of the retrieved articles was assessed by title and abstract, and conducted by two independent reviewers (MS and AJB). Studies that appeared to meet the inclusion criteria were selected for full-text review. Disagreements between the two independent reviewers were resolved by discussion.

We contacted the principal investigators of all eligible trials and requested individual patient data for baseline characteristics, tumour characteristics, preoperative treatment, surgery, adjuvant treatment, and follow-up.

## OUTCOMES

The primary end-point was overall survival. Secondary end-points were disease-free survival, and distant recurrences. All time-to-event variables were calculated from date of surgery. Overall survival was defined as time to death from any cause, or to end of follow-up (censored). Disease-free survival was defined as time to any recurrence or death, whichever occurred first, or end of follow-up (censored). Time to distant recurrence was defined as time to distant recurrence or end of follow-up (censored). The absence or presence of distant recurrence was confirmed by histology, cytology, or imaging.

## STATISTICAL ANALYSIS

To improve comparability between patients in the eligible trials, we included patients

with (y)pTNM stage II or III, who had a R0 resection, had a low anterior resection or an abdominoperineal resection, and had a tumour located ≤15 cm from the anal verge for the analysis. A sensitivity analysis of the primary end-point was performed in all patients who were originally included in the eligible trials.



Data were analysed for all included patients, as well as for the following patient subgroups: (y)pTNM stage (II *vs* III), tumour location from anal verge (<5 cm *vs* 5-9·9 cm *vs* ≥10 cm), type of resection (LAR *vs* APR), nodal status ((y)pN0 *vs* (y)pN1 *vs* (y)pN2), and preoperative treatment (short-course radiotherapy *vs* long-course radiotherapy *vs* long-course chemoradiotherapy).

The hazard ratio (HR) and 95% confidence interval (CI) for overall survival, disease-free survival, and the cause-specific hazard of distant recurrence, were calculated with Cox proportional hazards regression. The regression models included strata defined by a term representing the distinct trials. The cumulative incidence of distant recurrences was calculated with death as competing risk.[18] Median follow-up was calculated according to the reverse Kaplan-Meier method.[19] We did an interaction test of treatment efficacy with every subgroup for all outcome measures. Furthermore, analysis of the primary end-point was performed by trial, with all patients who were originally included in the eligible trials. These HRs and CIs slightly differ from the original articles, because more recent follow-up information was used.

The $I^2$ statistic, that should be interpreted "as the proportion of total variation in the estimates of treatment effect that is due to heterogeneity between studies", was calculated.[20] Furthermore, the Q statistic was calculated to assess if significant heterogeneity between the included trials existed.

The findings of our meta-analysis are presented in forest plots, with HRs and 95% CIs for all patients and for the above-mentioned subgroups of patients.

Statistical analyses were performed using IBM SPSS Statistics, version 20·0, and R, version 3·1·0. A p-value of 0·05 or less was considered as statistically significant.

## ROLE OF THE FUNDING SOURCE

The funding sources had no role in the study design, management, data analysis, and data interpretation. AJB, MS, HP, and CJHvdV had access to all study data. The corresponding author had the final responsibility for the decision to submit for publication.

# RESULTS



Our initial search identified 1131 citations. We excluded 1035 citations by title because they did not meet eligibility criteria. We read the abstracts of the remaining 96 articles. Of these, three full-text randomised controlled trials were read.[7, 13, 16] Furthermore, we found one eligible trial that was presented during the 29th European Society for Radiotherapy and Oncology (ESTRO) congress in 2010[21], and one abstract that was presented during the European Cancer Congress in 2013.[22] After contacting the principal investigators of these five studies, we obtained individual patient data of the I-CNR-RT trial, the Chronicle trial, the PROCTOR-SCRIPT trial (CJHvdV, corresponding author, is principal investigator), and the EORTC 22921 trial (Figure 1).[7, 15-17] Table 1 shows the main characteristics of these trials. The risk of bias of all included studies was judged as low. Although none of the studies was blinded, we think this has not influenced the outcome measurements.

**Table 1**. Study characteristics

| | PROCTOR/SCRIPT | EORTC 22921 | Chronicle | Italian study |
|---|---|---|---|---|
| **Neo-adjuvant treatment** | | | | |
| **Chemoradiotherapy** | 25x1.8-2 Gy + 5-FU based chemotherapy<br>5x5Gy | 25x1.8Gy + 5-FU based chemotherapy | 45 Gy + 5-FU based chemotherapy | 25x1.8Gy + 5-FU based chemotherapy |
| **Radiotherapy** | | 25x1.8Gy | | |
| **Adjuvant treatment** | Mayo regime: 6 courses of 5-FU (425mg/m²) and Folinic Acid (20mg/m²)<br>Nordic regime:12 courses of 5-FU (500mg/m²) and Folinic Acid (60mg/m²)<br>8 courses every three weeks of oral capecitabine (1250mg/m²) twice daily for 14 days | 4 courses every three weeks of 5-FU (350mg/ m²) and Folinic Acid (20mg/ m²) | 6 courses every three weeks of oxaliplatin (130mg m²) and oral capecitabine (1000mg/m²) twice daily for 14 days (XELOX) | 6 courses of 5-FU (350mg/ m²) and Folinic Acid (20mg/ m²) |
| **Start of accrual** | March 2000 | April 1993 | November 2004 | September 1992 |
| **End of accrual** | January 2013 | March 2003 | April 2008 | January 2001 |
| **Disease stage** | (y)pTNM II, III | Clinical stage T3,T4 | (y)pTNM II,III | Clinical stage T3,T4 |
| **Resection margin** | R0,R1 | R0 | R0 | R0 |
| **TME resection performed** | Yes | Halfway of the inclusion | Yes | No |
| **Timing of randomisation** | After surgery | Before surgery | After surgery | Before surgery |
| **Number of patients eligible for analysis (original study)** | 437 | 1011 | 113 | 634 |
| **Number of patients eligible for analysis in this article** | 403 | 473 | 75 | 245 |

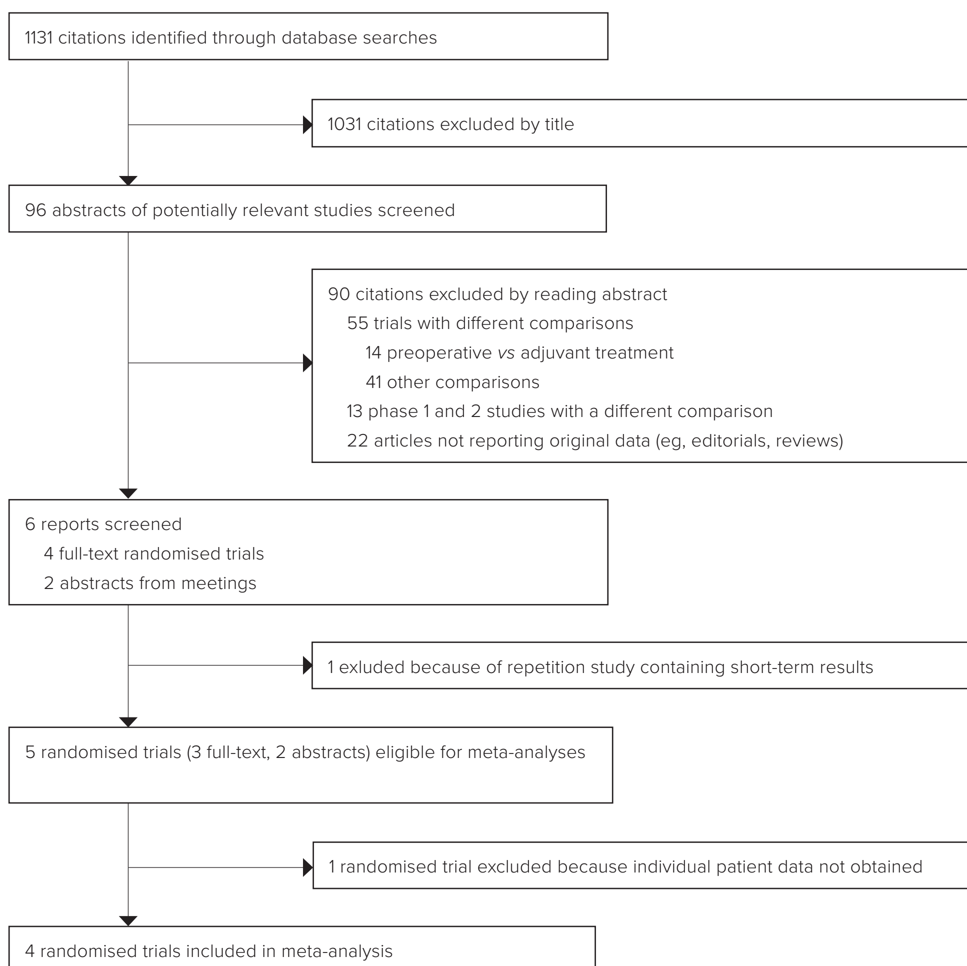


**Figure 1.** Selection of eligible trials

In total, there were 2195 patients included in four trials. To improve comparability, we selected 1196 patients for the analysis with (y)pTNM stage II or III, who had a R0 resection, had a low anterior resection or an abdominoperineal resection, and had a tumour located ≤15cm from the anal verge.

Of these 1196 patients, 598 patients had observation after surgery, and 598 patients received adjuvant chemotherapy. Patient characteristics are shown in Table 2. Median follow-up was 7·0 years (range: 0·0 - 17·4 years; two patients died on day of surgery).

**Table 2**. Patient characteristics

| Characteristics | Total (n = 1196) | | Observation (n =598) | | Chemotherapy (n =598) | |
|---|---|---|---|---|---|---|
| Trial | | | | | | |
| Italian | 245 | (20.5) | 112 | (18.7) | 133 | (22.2) |
| PROCTOR-SCRIPT | 403 | (33.7) | 204 | (34.1) | 199 | (33.3) |
| Chronicle | 75 | (6.3) | 45 | (7.5) | 30 | (5.0) |
| EORTC 22921 | 473 | (39.5) | 237 | (39.6) | 236 | (39.5) |
| Age (years) | 61.50 | ±9.60 | 62.00 | ±9.63 | 61.00 | ±9.57 |
| Gender | | | | | | |
| Male | 810 | (67.7) | 410 | (68.6) | 400 | (66.9) |
| Female | 386 | (32.3) | 188 | (31.4) | 198 | (33.1) |
| Preoperative treatment | | | | | | |
| 25 Gy | 348 | (29.1) | 179 | (29.9) | 169 | (28.3) |
| 45 Gy | 267 | (22.3) | 134 | (22.4) | 133 | (22.2) |
| 45 Gy + FU based chemo-therapy | 581 | (48.6) | 285 | (47.7) | 296 | (49.5) |
| Type of resection | | | | | | |
| LAR | 726 | (60.7) | 362 | (60.5) | 364 | (60.9) |
| APR | 470 | (39.3) | 236 | (39.5) | 234 | (39.1) |
| Tumour location from anal verge | | | | | | |
| < 5 cm | 381 | (31.9) | 187 | (31.3) | 194 | (32.4) |
| 5 – 9.9 cm | 519 | (43.4) | 256 | (42.8) | 263 | (44.0) |
| ≥ 10 cm | 281 | (23.5) | 144 | (24.1) | 137 | (22.9) |
| Unknown | 15 | (1.3) | 11 | (1.8) | 4 | (0.7) |
| (y)pTNM | | | | | | |
| II | 459 | (38.4) | 207 | (34.6) | 252 | (42.1) |
| III | 737 | (61.6) | 391 | (65.4) | 346 | (57.9) |

Data are presented as median ± SD or as *n* (%)

## OVERALL SURVIVAL

A total of 451 patients died. Figure 2A shows a forest plot of hazard ratios for overall survival for all patients and for subgroups. Overall, no benefit in overall survival was observed for patients who received adjuvant chemotherapy compared with observation (HR 0·97, 95% CI 0·81-1·17, p=0·775). Also in subgroup analysis, no significant differences in overall survival were found. Sensitivity analysis of all 2195 patients showed a HR of 0·95 (95% CI 0·82-1·09, p=0·430). Supplementary Figure 1 shows a forest plot of hazard ratios for overall survival by study.

We found no heterogeneity in treatment effect between the four trials ($I^2$=0%, p=0·605).

## DISEASE-FREE SURVIVAL

In total, there were 580 events. The disease-free survival results are shown in Figure 2B. Overall, we observed no statistically significant difference in disease-free survival for

patients who received adjuvant chemotherapy compared with observation (HR 0·91, 95% CI 0·77-1·07, p=0·230). In subgroup analysis, patients with a tumour between 10 cm and 15 cm from the anal verge who received adjuvant chemotherapy had an improved disease-free survival (HR 0·59, 95% CI 0·40-0·85, p=0·005), without a significant interaction between distance from the anal verge (<5 cm *vs* 5-9·9 cm *vs* ≥10 cm) and randomisation arm (p=0·107). For the other subgroups, there were no differences in disease-free survival.

There was no heterogeneity of adjuvant chemotherapy effect among the four trials ($I^2$=0%, p=0·836).

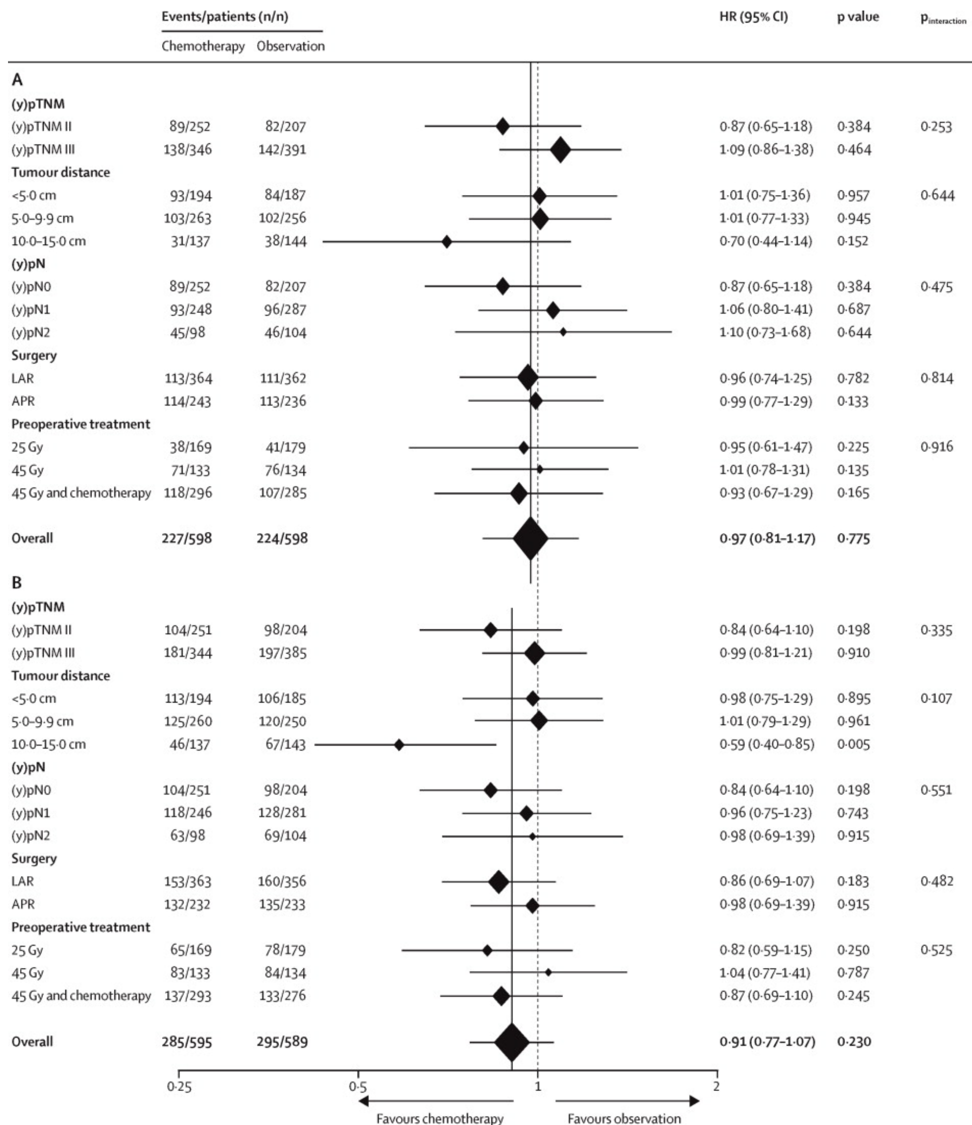


**Figure 2.** Overall survival (A) and disease-free survival (B) for all patients and by patient subgroups

Footnote Figure 2: The size of the diamonds represents the proportion of patients

## DISTANT RECURRENCE

There were 415 distant recurrences. Overall, we did not observe a significant benefit of adjuvant chemotherapy. At five years, the cumulative incidence for distant recurrences was 36·51% (95% CI 32·64%-40·84%) in the observation arm and 35·50% (95% CI 31·70%-39·76%) in the chemotherapy arm (HR 0·94, 95% CI 0·78-1·14, p=0·523; Figure 3; Figure 4). However, patients with a tumour between 10 cm and 15 cm from the anal verge showed a benefit of adjuvant chemotherapy with regard to distant recurrence (HR 0·61, 95% CI 0·40-0·94, p=0·025), without a significant interaction between distance from the anal verge and randomisation arm (p=0·126). Similar to disease-free survival, there were no significant differences for the other subgroups between observation and adjuvant chemotherapy (Figure 3).

We found no heterogeneity in treatment effect between the four trials ($I^2$=0%, p=0·617).

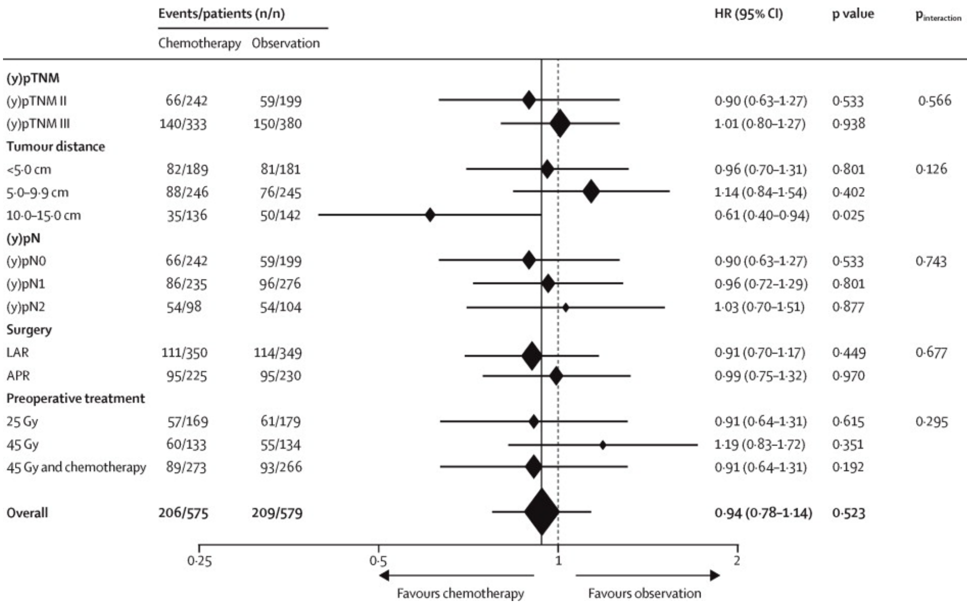


**Figure 3.** Distant recurrence

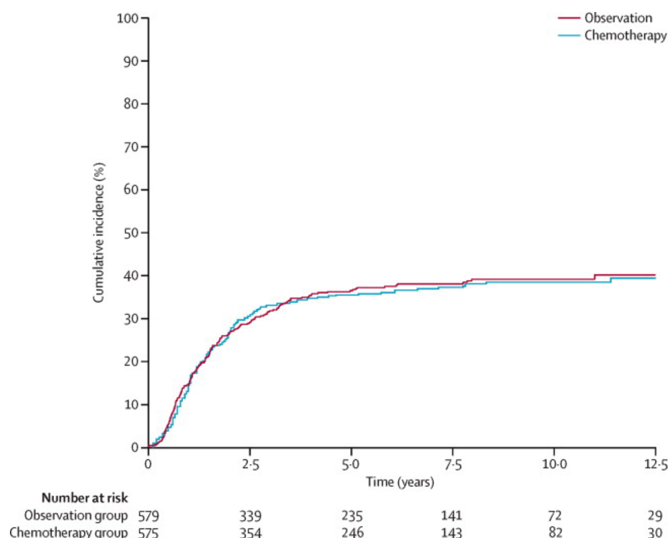


**Figure 4** Cumulative incidence of distant recurrences

# DISCUSSION

This meta-analysis pooled individual patient data of four randomised controlled trials comparing observation with adjuvant 5-FU based chemotherapy after preoperative (chemo)radiotherapy and surgery for patients with rectal cancer. Overall, no benefit of 5-FU based adjuvant chemotherapy was shown with regard to overall survival, disease-free survival, and distant recurrences after a median follow-up of 7·0 years. However, our findings suggest that patients with a tumour located between 10 cm and 15 cm from the anal verge may benefit from adjuvant chemotherapy in terms of disease-free survival and distant recurrences.

Although a clear benefit of adjuvant chemotherapy has been demonstrated for patients with stage III colon cancer[23-26], this is not the case for patients with non-metastatic rectal cancer treated with preoperative (chemo)radiotherapy and surgery. The inconclusive evidence on the use of adjuvant chemotherapy after preoperative (chemo)radiotherapy and surgery for patients with rectal cancer is reflected by international differences in guidelines varying from not recommending adjuvant chemotherapy to recommending adjuvant chemotherapy for stage II and III rectal cancer patients.[27-30] The latter is based on extrapolation from phase III trials for adjuvant treatment for colon cancer[23-26], as well as from trials in patients with rectal cancer who were treated without preoperative (chemo)radiotherapy.[11]

However, even though four out of five European randomised controlled trials comparing adjuvant chemotherapy with observation after receiving preoperative (chemo)radiotherapy and surgery did not demonstrate a clinical relevant or statistical significant benefit of adjuvant chemotherapy[7, 15-17], none has individually put an end to the discussion on the role of adjuvant chemotherapy. This could be partly explained by the fact that two of these trials did not have sufficient power.[15, 16] Only the QUASAR trial found a borderline significant improvement in overall survival for patients with rectal cancer who were randomised to adjuvant chemotherapy, but only 21% of patients with rectal cancer or both rectal and colon cancer had preoperative radiotherapy and no patient received chemoradiotherapy.[13]

By pooling the individual patient data from the I-CNR-RT trial, the EORTC 22921 trial, the Chronicle trial, and the PROCTOR-SCRIPT trial[7, 15-17], we think this meta-analysis is the most robust analysis of the role of adjuvant 5-FU based chemotherapy for patients with rectal cancer to date, enabling to increase the statistical power, to improve comparability between the patients in the four individual trials, and to perform subgroup analysis.

Besides the embryological, anatomical, and physiological differences between colon and rectum, accumulating evidence suggests that colon and rectal cancer differ in oncogenesis.[31] Differences include reduced microsatellite instability (MSI) and BRAF mutations in rectal cancer compared with colon cancer.[32-34] Furthermore, in the last decade, different gene expression profiles between colon and rectal tumours, as well as within the colon were observed.[35, 36] These differences between colon and rectal tumours might contribute to the differences in beneficial effect of adjuvant chemotherapy between colon and rectal cancer. In contrast, no clear differences in *KRAS* mutations between colon and rectal tumours were demonstrated.[37-40]

Interestingly, despite the suggestion that colon and rectal tumours differ in carcinogenesis, the definition of the rectum is not consistent across countries with regard to distance from the anal verge and location of the peritoneal reflection. Although the results of our meta-analysis overall do not demonstrate a benefit of adjuvant chemotherapy in overall survival, disease-free survival, and distant recurrences, our results suggest that patients with a tumour between 10 cm and 15 cm from the anal verge may benefit from adjuvant chemotherapy in terms of disease-free survival and distant recurrences. This raises the question whether tumours between 10 cm and 15 cm from the anal verge should be defined as colon tumours rather than rectal tumours, that may require other treatment approaches than rectal tumours below 10 cm from the anal verge. However, since there is no significant interaction between distance

from the anal verge and randomisation arm, these results are not definitive. Further investigation with regard to preoperative and postoperative treatment for patients with a tumour between 10 cm and 15 cm from the anal verge is warranted to draw definitive conclusions for these patients. In contrast, no benefit of adjuvant chemotherapy was demonstrated for other subgroups. Unfortunately, patients with ypTNM 0 and ypTNM I were only included in the I-CNR-RT trial, and partly in the EORTC 22921 trial. Therefore, it was not possible to perform a meta-analysis on ypTNM stage 0 and ypTNM stage I, although this would have been interesting.



An individual patient data meta-analysis has advantages over an aggregate data meta-analysis, as for example the possibility to obtain results for specific subgroups.[41] Although we think this individual patient data meta-analysis on the effect of adjuvant chemotherapy in rectal cancer patients after preoperative (chemo)radiotherapy and surgery provides the best current available evidence, this study has some limitations. A well-recognised problem in randomised controlled trials is to obtain sufficient power.[42] Patients´ and clinicians´ treatment preferences for either observation or adjuvant chemotherapy, contributed to the fact that two of the included trials in this meta-analysis had to close their study before the intended number of patients was reached.[15, 16] Another well-known problem of trials investigating the role of adjuvant chemotherapy in patients with rectal cancer after preoperative (chemo)radiotherapy and surgery is adjuvant chemotherapy compliance. In the PROCTOR-SCRIPT trial, adjuvant chemotherapy compliance was 73.6% (randomisation postoperatively).[15] In the EORTC 22921 trial (randomisation preoperatively) 43% completed all cycles of chemotherapy[7], while this amounted 48% in the Chronicle trial (randomisation postoperatively).[16] In the I-CNR-RT trial (randomisation preoperatively), 55% received three to six courses chemotherapy.[17] In theory, this could have influenced the results, although we think it is unlikely that this has influenced the overall outcomes significantly. For example, in the per-protocol analysis of the PROCTOR-SCRIPT trial[15], no benefit of adjuvant chemotherapy was demonstrated in patients who completed all cycles of adjuvant chemotherapy. Besides, the EORTC 22921 trial, the I-CNR-RT trial, and the PROCTOR-SCRIPT trial all had a long accrual period. For example, TME surgery was not yet standard of care during the greatest part of the inclusion period of the I-CNR-RT trial, and became standard of care halfway the inclusion period of the EORTC 22921 trial. Lastly, the QUASAR trial is not included in our meta-analysis, because we unfortunately did not obtain the individual patient data.

If patients with a tumour between 10 cm and 15 cm from the anal verge indeed do benefit from adjuvant chemotherapy, the question is whether fluoropyrimidine monotherapy or combination chemotherapy should be administered. No clear evidence of superiority

of fluoropyrimidine monotherapy or combination chemotherapy existed at the start of most of the included trials. Three out of four trials included in this meta-analysis used fluoropyrimidine monotherapy.[7, 15-17] In 2009, the MOSAIC trial demonstrated an improved disease-free survival and overall survival for patients with colon cancer by adding oxaliplatin to 5-FU/LV.[26, 43] For this reason, the Chronicle trial administered combination chemotherapy.[16] Recently, the ADORE trial showed that there seems to be a benefit of adjuvant FOLFOX over 5-FU/LV for patients with ypTNM stage II or III rectal cancer.[44] Besides, the results of the CAO/ARO/AIO-04 trial (presented during the 2014 ASCO Annual Meeting) demonstrated a benefit of adjuvant combination chemotherapy over 5-FU monotherapy.[45] Because the lack of an observation arm in both studies, these studies were unfortunately not eligible in this meta-analysis. The question whether there is a benefit of adjuvant combination chemotherapy over observation remains unanswered.

In conclusion, overall, 5-FU based adjuvant chemotherapy did not improve overall survival, disease-free survival and distant recurrences compared with observation in rectal cancer patients. However, our findings suggest that patients with a tumour located between 10 cm and 15 cm from the anal verge may benefit from adjuvant chemotherapy in terms of disease-free survival and distant recurrences. Further research with regard to preoperative and postoperative treatment for this subgroup of patients is warranted.

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# Chapter 3

## Should rectal cancer located 10-15cm from the anal verge be defined as colon cancer

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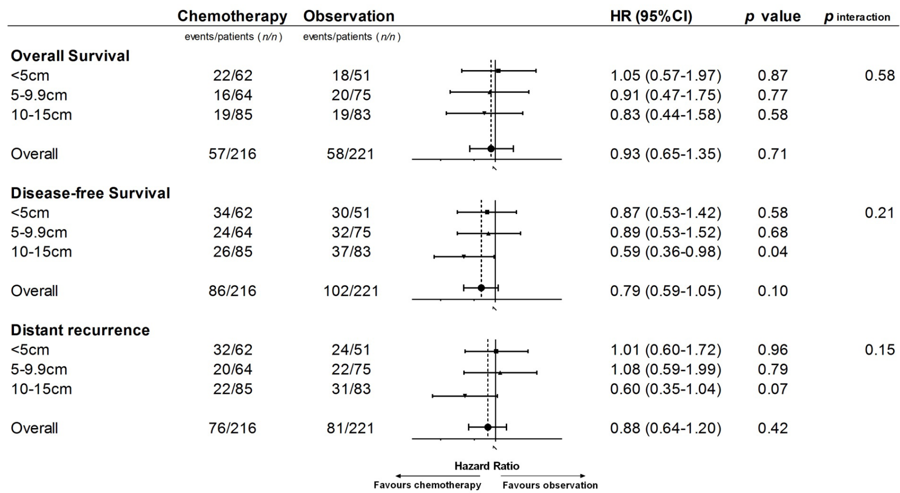
Because colon and rectal tumours biologically differ, a clear separation of colon and rectal cancer for scientific research and treatment strategies is needed. However, the definition of the rectum is inconsistent across countries regarding location of the peritoneal reflection and distance from the anal verge. A recently published meta-analysis on individual patient data demonstrated that adjuvant chemotherapy after preoperative (chemo)radiotherapy and TME surgery did not improve overall survival, disease-free survival (DFS) and distant recurrence rates in patients with pathological stage II-III rectal cancer [1,2]. In the meta-analysis it was suggested that a subgroup of patients with rectal tumours 10-15cm from the anal verge might benefit from adjuvant chemotherapy in terms of DFS and distant recurrences [2]. Consequently, it could be debated whether tumours located 10-15cm from the anal verge should be defined as colon tumours rather than rectal tumours, since patients with stage III and high-risk stage II colon cancer do benefit from adjuvant chemotherapy [3]. Further investigation for patients with rectal tumours 10-15cm from the anal verge is essential, although a randomized trial is not feasible. Therefore, we report on the results of the PROCTOR/SCRIPT trial after a median follow-up of 5.5 years, with a focus on rectal tumours 10-15cm from the anal verge. In this study, a multicenter randomized phase III trial, patients were randomly assigned to adjuvant chemotherapy or observation in patients with (y)pTNM stage II-III rectal cancer treated with preoperative (chemo)radiotherapy and TME surgery. Study design, patient characteristics, definitions of endpoints and exclusion criteria were described elsewhere [1]. In agreement with the previous reported results with a median follow-up of 5 years, no beneficial effect of adjuvant treatment was observed in the total study cohort (*N*=437). However, a significant benefit in DFS (HR 0.59, 95% CI;0.36-0.98, *P*=0.04) was observed in patients randomised to adjuvant chemotherapy for (y)pTNM stage II-III rectal cancer located 10-15cm of the anal verge treated with preoperative (chemo)radiotherapy and TME surgery (figure). This beneficial effect has not been observed in patients with tumours located <5cm and 5-9.9cm from the anal verge (Figure 1). No significant interaction between distance from the anal verge and treatment group was detected. We acknowledge that the PROCTOR/SCRIPT trial was not powered to perform subgroup analysis. Based on the meta-analysis, supported by our updated data, we propose that tumours located 10-15cm from the anal verge might be defined as colon tumours instead of rectum tumours considering the suggested beneficial effect on DFS of adjuvant chemotherapy.



| | Chemotherapy events/patients (*n/n*) | Observation events/patients (*n/n*) | HR (95%CI) | *p* value | *p* interaction |
|---|---|---|---|---|---|
| **Overall Survival** | | | | | |
| <5cm | 22/62 | 18/51 | 1.05 (0.57-1.97) | 0.87 | 0.58 |
| 5-9.9cm | 16/64 | 20/75 | 0.91 (0.47-1.75) | 0.77 | |
| 10-15cm | 19/85 | 19/83 | 0.83 (0.44-1.58) | 0.58 | |
| Overall | 57/216 | 58/221 | 0.93 (0.65-1.35) | 0.71 | |
| **Disease-free Survival** | | | | | |
| <5cm | 34/62 | 30/51 | 0.87 (0.53-1.42) | 0.58 | 0.21 |
| 5-9.9cm | 24/64 | 32/75 | 0.89 (0.53-1.52) | 0.68 | |
| 10-15cm | 26/85 | 37/83 | 0.59 (0.36-0.98) | 0.04 | |
| Overall | 86/216 | 102/221 | 0.79 (0.59-1.05) | 0.10 | |
| **Distant recurrence** | | | | | |
| <5cm | 32/62 | 24/51 | 1.01 (0.60-1.72) | 0.96 | 0.15 |
| 5-9.9cm | 20/64 | 22/75 | 1.08 (0.59-1.99) | 0.79 | |
| 10-15cm | 22/85 | 31/83 | 0.60 (0.35-1.04) | 0.07 | |
| Overall | 76/216 | 81/221 | 0.88 (0.64-1.20) | 0.42 | |



**Figure 1.** Overall survival, disease-free survival ans distant recurrence for all patients and by patient subgroups

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# Chapter 4

## Are pathological high risk features in locally advanced rectal cancer a useful selection tool for adjuvant chemotherapy?

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# ABSTRACT

### BACKGROUND

Several histological high risk factors are used as an indication for adjuvant therapy in stage II colon cancer. Those and other factors, including lymphatic invasion, perineural invasion, venous invasion and tumour budding are associated with decreased outcome. In this study, we evaluated the prognostic and predictive value of these biomarkers in a cohort of rectal cancer patients.

### MATERIALS AND METHODS

The trial-based cohort consisted of 221npTNM stage II-III rectal cancer patients, included in the PROCTOR/SCRIPT trial, a multicentre randomized phase III trial. Patients treated with neoadjuvant radiotherapy and TME surgery, were randomized between adjuvant chemotherapy or observation. Lymphatic invasion, perineural invasion, extramural venous invasion, intramural venous invasion and tumour budding was determined in standard tissue slides.

### RESULTS

The presence of perineural invasion (HR 3.36; 95%CI 1.82-6.21), extramural vascular invasion (HR 1.93; 95%CI 1.17-3.19), and tumour budding (HR 1.83, 95%CI 1.11-3.03) was associated with a significant worse overall survival. The presence of $\geq$ 2 adverse biomarkers resulted in a stronger prediction of adverse outcome in terms of overall survival (HR 2.82; 95%CI 1.66-4.79), disease-free survival (HR 2.27; 95%CI 1.47-3.48) and distant recurrence (HR 2.51; 95%CI 1.56-4.02). None of these markers alone or combined predicted a beneficial effect of adjuvant chemotherapy.

### DISCUSSION

We confirmed that several stage independent biomarkers were significantly associated with a decreased outcome in rectal cancer patients. More importantly, these markers did not have predictive value, and are thus no useful to select for adjuvant therapy in rectal cancer.

# INTRODUCTION

Treatment regimens in patients with rectal cancer are primarily influenced by the tumour, node and metastasis (TNM) classification and the circumferential resection margin, which provide an estimation of the patient's prognosis [1]. Pathological staging is essential for planning the most appropriate treatment in patients with rectal cancer, however outcome among patients with the same tumour stage differs significantly [2]. Consequently, it could be stated that conventional classification does not provide adequate individualized assessment.

For patients with stage III or high risk stage II colon tumours, adjuvant chemotherapy is indicated after surgery [3,4]. The high risk stage II colon tumour is mainly defined by histopathologic characteristics such as the presence of a T4 tumour, extramural vascular invasion (EMVI), poor differentiation, less than 10 harvested lymph nodes or patients who have had obstruction or perforation [3-5]. In order to optimize the delivery of adjuvant chemotherapy in rectal cancer, additional histological risk factors should be explored. Those factors, include lymphatic invasion, perineural invasion (PNI), EMVI, intramural venous invasion (IMVI) and tumour budding which are all associated with decreased clinical outcome [6-12]. In the seventh edition of the TNM, these items were included as accessory markers because of their relevance [1]. It has been proposed that these biomarkers may guide treatment decisions, particular the use of adjuvant chemotherapy [10,13,14]. Thus, in contrast with colon cancer, the benefit of adjuvant chemotherapy in rectal cancer has not been demonstrated [15,16]. In the future improvements of the patient selection might reveal high risk rectal cancer patients who do benefit from adjuvant chemotherapy. Therefore, the proposed prognostic [10,13,14] and predictive value of the above mentioned biomarkers was evaluated on standard tissue slides, of patients with stage II-III locally advanced rectal cancer included in the PROCTOR-SCRIPT trail.

# MATERIALS AND METHODS

## PATIENT SELECTION

Data were derived from patients included in the PROCTOR-SCRIPT trial (ISRCTN; 36266738), a multicentre randomized phase III trial, that included patients with (y)pTNM stage II-III rectal cancer treated with neoadjuvant (chemo)radiotherapy and TME surgery, randomly assigned to adjuvant chemotherapy or observation. The results of the primary and secondary endpoints have been published previously [15]. Informed consent for

participation and retrospective use of samples was obtained from all patients. Formalin-fixed paraffin-embedded (FFPE) tumour samples of the included Dutch patients were collected. Only patients treated with neoadjuvant radiotherapy (5x5Gy) were included in this analysis. Patients treated with neoadjuvant chemoradiotherapy were excluded in order to establish a cohort with similar neoadjuvant treatment regimes.

## PATHOLOGICAL ASSESSMENT

Standardized pathological examination according to Quirke *et al.* was performed in the laboratories of the referring hospitals [17]. FFPR tumour tissue sections of 4 μm were stained with Haematoxylin and Eosin. All tumour sections were reviewed by a single pathologist (I.D.N.) for the presence or absence of lymphatic invasion, PNI, EMVI, IMVI, and tumour budding. Lymphatic invasion was defined as the presence of tumour cells within an endothelial-lined lymphatic channel. PNI was defined as, tumour cells growing around, within and through any of the three nerve layers and should surround more than 33% of the nerve circumference [11]. Venous invasion was defined as tumour cells within an area lined by endothelial and smooth muscle cells or elastic fibres. Venous invasion was divided in IMVI and EMVI, whereas EMVI was venous invasion located outside the muscularis propria within the surrounding mesorectal fat [18]. Thereby, the presence of an adjacent arterial structure was required. Tumour budding was evaluated as positive when small clusters of tumour cells, fewer than five undifferentiated tumour cells, were observed at the invasive front [19]. The impact of these factors on outcome was analysed separately for each factor and in combination, where patients with none or one biomarker present were compared to patients with two or more biomarkers present.



## STATISTICAL ANALYSIS

Statistical analyses were performed using the statistical package SPSS (version 20.0 for Windows; SPSS Inc.). The Student T-test and Chi-square test were used to evaluate association between the biomarkers, and combinations thereof, and clinico-pathological parameters.

Overall survival (OS) was defined as time since randomization until death. Disease-free survival (DFS) was defined as time since randomization until local recurrence, distant recurrence or death, whichever came first. Time to distant recurrence (DR) was defined as time to distant metastasis, or end of follow-up, deaths were censored in this analysis. For survival probabilities the Kaplan-Meier method was used and for comparison of survival curves the Log-Rank test was used. Univariate and multivariate Cox regression analyses were performed to evaluate the differences in OS, DFS and DR. Covariates entered in the multivariate model were age, gender, stage and circumferential resection margin. For all tests a p-value of $<0.05$ was considered as statistically significant.

# RESULTS

## PATIENT CHARACTERISTICS

In total 470 patients were enrolled in the PROCTOR-SCRIPT trial (146 Swedish and 324 Dutch patients). Only tumour tissue of Dutch patients was available for this study, and was successfully obtained for 262 patients, of whom 11 were ineligible. In order to establish a homogenous cohort, patients treated with neoadjuvant chemoradiotherapy were excluded (n=30). This resulted in a total study cohort of 221 patients with locally advanced rectal cancer, treated with neoadjuvant short-course radiotherapy (5x5Gy) Figure 1. Of the eligible patients, 104 patients were randomized assigned to adjuvant chemotherapy and 117 patients to observation, with a median follow-up of 5.4 years for the total cohort. Patients characteristics of the study cohort are summarized in Table 1. As shown in this table the presence of ≥ 2 biomarkers was associated with a higher disease stage.



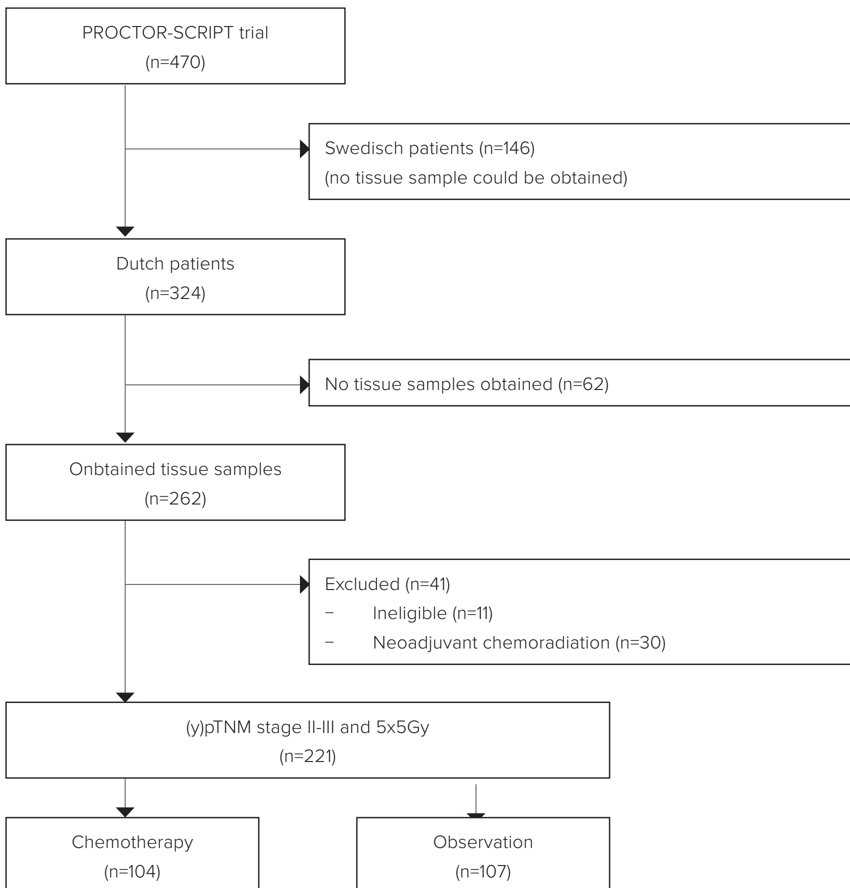


**Figure 1:** Patient selection.

**Table 1:** Patient characteristics of the total study cohort and stratified for < 2 and ≥ 2 adverse biomarkers.

| | Total population | | Adverse biomarkers < 2 biomarkers | | Adverse biomarkers ≥ 2 biomarkers | | |
|---|---|---|---|---|---|---|---|
| | n=221 | (%) | n=118 | (%) | n=103 | (%) | P-value |
| Age median | 59.8 | (±9.7) | 58.7 | (±9.4) | 61.0 | (±9.9) | 0.10 |
| Gender | | | | | | | |
| Male | 136 | (61.5) | 72 | (61.0) | 64 | (62.1) | 0.86 |
| Female | 85 | (38.5) | 46 | (39.0) | 39 | (37.9) | |
| Tumour location from the anal verge | | | | | | | |
| < 5 cm | 61 | (27.6) | 29 | (24.6) | 32 | (31.1) | 0.49 |
| 5-9.9 cm | 69 | (31.2) | 40 | (33.9) | 29 | (28.2) | |
| >10 cm | 85 | (38.5) | 46 | (39.0) | 39 | (37.9) | |
| Unknown | 6 | (2.7) | 3 | (2.5) | 3 | (2.9) | |
| (y)pTNM | | | | | | | |
| II | 29 | (13.1) | 21 | (17.8) | 8 | (7.8) | 0.03 |
| III | 192 | (86.9) | 97 | (82.2) | 95 | (92.2) | |
| Differentiation | | | | | | | |
| Well | 5 | (2.3) | 4 | (3.4) | 1 | (1.0) | 0.05 |
| Moderate | 196 | (88.7) | 107 | (90.7) | 89 | (86.4) | |
| Poor | 18 | (8.1) | 5 | (4.2) | 13 | (12.6) | |
| Unknown | 2 | (0.9) | 2 | (1.7) | 0 | (0.0) | |
| CRM | | | | | | | |
| Negative | 208 | (94.1) | 110 | (93.2) | 98 | (95.1) | 0.55 |
| Positive | 13 | (5.9) | 8 | (6.8) | 5 | (4.9) | |
| Adjuvant treatment | | | | | | | |
| Chemotherapy | 104 | (47.1) | 58 | (49.2) | 46 | (44.7) | 0.50 |
| Observation | 117 | (52.9) | 60 | (50.8) | 57 | (55.3) | |

## SINGLE BIOMARKERS IN RELATION TO PATIENT OUTCOME

As shown in Figure 2, the presence of PNI (HR 3.36; 95%CI 1.82-6.21), EMVI (HR 1.93; 95%CI 1.17-3.19), and tumour budding (HR 1.83; 95%CI 1.11-3.03) was significantly associated with a decreased overall survival in the univariate analysis. For lymphatic invasion (HR 1.61; 95%CI 0.96-2.70) and IMVI (HR 1.30; 95%CI 0.71-2.40) trends towards a worse prognosis could be observed. In the multivariate analysis, corrected for age, gender, stage and, circumferential resection margin status, the effects remained significant for PNI (HR 2.68; 95%CI 1.41-5.11), EMVI (HR 2.08; 95%CI 1.26-3.46) and tumour budding (HR 1.54; 95%CI 1.09-3.03). As shown in Table 2, significantly worse DFS and a higher distant recurrence rates were observed in patients with PNI, EMVI, IMVI, and, tumour budding.

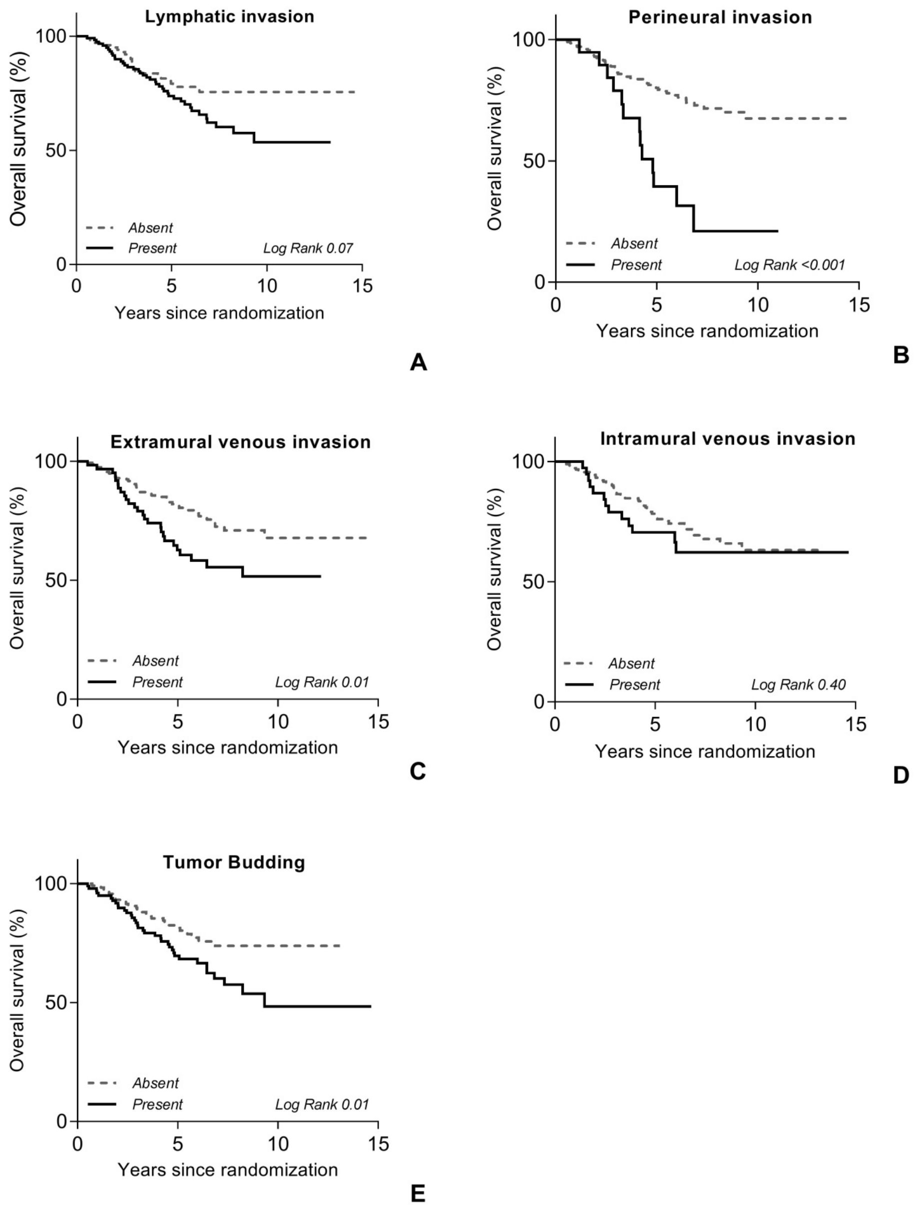


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**Figure 2**: Kaplan-Meier analysis for overall survival in 221 stage II-III rectal cancer patients according to the status of the different tumour parameters, dichotomized as present or absent. The p-value represents the Log-Rank test. (A) Lymphatic invasion. (B) Perineural invasion. (C) Extramural vascular invasion. (D) Intramural vascular invasion. (E) Tumour budding.

**Table 2.** Univariate and multivariate survival analysis for disease-free survival and time to distant recurrence according to different pathological factors. Covariates entered in the multivariate model were age, gender, stage and circumferential resection margin status.

| | | Disease-free Survival | | | | Time to distant recurrence | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Univariate | | Multivariate | | Univariate | | Multivariate | |
| | Patients (%) | HR (95%CI) | P-value | HR (95%CI) | P-value | HR (95%CI) | P-value | HR (95%CI) | P-value |
| **Lymphatic invasion** | | | | | | | | | |
| Absent | 100 (45) | 1.00 | 0.19 | 1.00 | 0.24 | 1.00 | 0.31 | 1.00 | 0.36 |
| Present | 120 (55) | 1.33 (0.87-2.05) | | 1.30 (0.84-2.04) | | 1.27 (0.80-2.01) | | 1.25 (0.78-2.00) | |
| **Perineural invasion** | | | | | | | | | |
| Absent | 202 (91) | 1.00 | 0.001 | 1.00 | 0.007 | 1.00 | <0.001 | 1.00 | 0.001 |
| Present | 19 (9) | 2.63 (1.46-4.76) | | 2.33 (1.26-4.31) | | 3.09 (1.69-5.64) | | 2.91 (1.56-5.45) | |
| **Extramural vascular invasion** | | | | | | | | | |
| Absent | 159 (72) | 1.00 | 0.04 | 1.00 | 0.03 | 1.00 | 0.03 | 1.00 | 0.02 |
| Present | 62 (28) | 1.59 (1.03-2.45) | | 1.64 (1.06-2.55) | | 1.66 (1.04-2.66) | | 1.76 (1.10-2.83) | |
| **Intramural vascular invasion** | | | | | | | | | |
| Absent | 182 (83) | 1.00 | 0.02 | 1.00 | 0.07 | 1.00 | 0.008 | 1.00 | 0.03 |
| Present | 38 (17) | 1.79 (1.09-2.92) | | 1.60 (0.97-2.65) | | 2.01(1.20-3.35) | | 1.82 (1.08-3.08) | |
| **Tumor Budding** | | | | | | | | | |
| Absent | 119 (55) | 1.00 | 0.05 | 1.00 | 0.05 | 1.00 | 0.05 | 1.00 | 0.05 |
| Present | 99 (45) | 1.52 (0.99-2.32) | | 1.54 (1.00-2.37) | | 1.59 (1.00-2.52) | | 1.60 (1.00-2.57) | |

### COMBINED ANALYSIS OF BIOMARKERS IN RELATION TO PATIENT OUTCOME

To analyse the effect of multiple biomarkers on patient outcome, all single biomarkers (lymphatic invasion, PNI, EMVI, IMVI, tumour budding) were combined, and stratified in two groups. The first group consisted of patients with none or just one biomarker was observed to be present in the tumour tissue section. The second group consists of patients with the presence of ≥ 2 biomarkers present. As shown in Figure 3, patients with ≥2 adverse biomarkers had a significant worse OS ($p<0.001$) and DFS ($p<0.001$). The cumulative incidence for distant recurrence was 23% in patients with <2 adverse biomarkers 47% in patients with ≥2 adverse biomarkers ($p<0.001$). In the multivariate analysis, corrected for age, gender, stage and circumferential resection margin, a significant worse OS (HR 2.73; 95%CI 1.58-4.71), DFS (HR 2.30; 95%CI 1.48-3.59) and DR (HR 2.59; 95%CI 1.60-4.22) was observed in patients with ≥2 adverse biomarkers compared to the group of patients with <2 adverse biomarkers.



### EFFECT OF ADJUVANT CHEMOTHERAPY IN PATIENTS WITH ADVERSE PROGNOSTIC BIOMARKERS

No benefit of adjuvant chemotherapy was shown for any biomarker-based subgroup regarding OS (Figure 4). Similar results were obtained for DFS and DR (data not shown). When patients with <2 and ≥ 2 adverse biomarkers were evaluated for the effect of adjuvant chemotherapy, no statistically significant beneficial effect for the use of adjuvant chemotherapy could have been observed, (HR 1.46; 95%CI 0.59-3.56) and (HR 1.08; 95%CI 0.59-1.97) respectively (Figure 4). Thus, in patients with a significant worse overall (≥ 2 adverse biomarkers), no beneficial effect of adjuvant chemotherapy was observed.

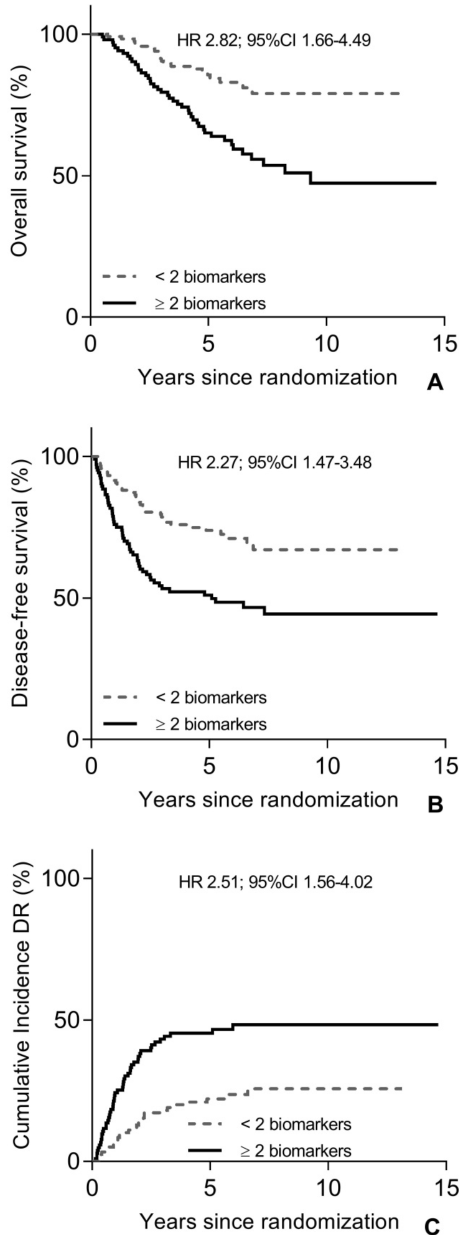


**Figure 3:** Survival curves for (A) overall survival, (B) disease-free survival and (C) cumulative incidence of distant recurrence, according to the presence of < 2 or ≥ 2 adverse biomarkers. Biomarkers include; lymphatic invasion, perineural invasion, extramural vascular invasion, intramural vascular invasion or tumour budding. Hazard ratio's (HR) and 95% confidence interval (95%CI) in the graph represents the univariate analysis.

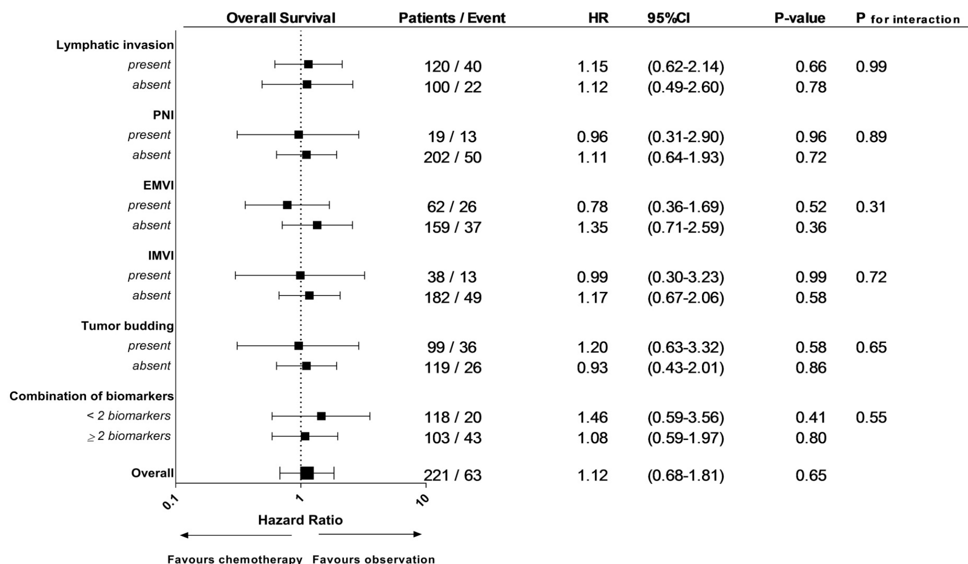


**Figure 4.** Overall survival for all patients and by patients subgroups, comparing observation and adjuvant chemotherapy.



# DISCUSSION

Standardized histopathological staging for rectal cancer is currently the cornerstone for prognostic assessment and highly influences rectal cancer treatment. In the current study, additional stage independent pathological markers were investigated. This study confirmed that, stage-independent pathological markers, including PNI, EMVI, IMVI, and tumour budding, were powerful tools for prognostication. Especially when all biomarkers were combined. The strong prognostic effect observed in rectal cancer patients of which the tumour showed the presence of ≥ 2 adverse biomarkers, could be explained by the access of multiple routes for metastatic spread. These tumour cells can disseminate through more than one route, blood, lymph channels or along nerves, and consequently could result in more extensive metastasis, as has been suggested before. [20]

In earlier studies, it was hypothesized that the investigated biomarkers were considered as good indicators for the use of adjuvant chemotherapy [7,10,21], since adjuvant chemotherapy might eliminate micrometastases and circulating tumour cells, preventing distant metastasis. However, when comparing the different rectal cancer patient subgroups based on these biomarkers, no beneficial effect of adjuvant

chemotherapy could be demonstrated in this randomised cohort comparing adjuvant chemotherapy with observation. More interestingly, no beneficial effect of adjuvant chemotherapy was observed in the patient group with the poorest prognosis, as indicated by the presence of two or more biomarkers. These findings were in line with the previously published overall results of the PROCTOR-SCRIPT trial and a more recently published meta-analysis, both demonstrating no beneficial effect of adjuvant chemotherapy in patients with locally advanced rectal cancer treated with neoadjuvant (chemo)radiotherapy and TME surgery [15,16].



Overall, the reported incidence of the investigated biomarkers varies in literature, most likely caused by the different criteria used for the detection. For example, in the present study 9.5% of the patients had PNI, which is lower than the recently reported incidence of 20.8% in rectal cancer [9]. However, neoadjuvant treatment is associated with less frequent PNI [9,22]. In the current study, EMVI was observed in 28% and IMVI was observed in 17.2% of the patient cohort, which is comparable to the incidence reported elsewhere [7]. IMVI was not significantly associated with survival. However, significantly more distant recurrences did occur in patients with IMVI. This is in line with the current literature, demonstrating a less clear relation of IMVI with survival compared to EMVI [6,18].

Although our findings are interesting, we acknowledge that the performed study has some limitations. First, our sample size for analysis performed in some subgroups were moderately sized. Secondly, standard tissue slides were used in this study. For more detailed evaluation additional immunohistochemical staining could be used for the investigated biomarkers. These limitations are exceeded by the strengths of this study, the large trial-based cohort, with prospectively collected patient data and the random allocation to observation or adjuvant chemotherapy. Furthermore, to the best of our knowledge this is the first study evaluating these pathological biomarkers in a rectal cancer cohort receiving a 5x5Gy as pre-operative treatment, in contrast with the study performed by Nikberg *et al* where 53% of the patients received neoadjuvant radiotherapy (17% neoadjuvant chemoradiotherapy and 20% no pre-operative therapy) [10]. Moreover, our study shows that, in rectal cancer, prognostic factors cannot as yet be used as predictive factors for adjuvant therapy. Therefore, we must be cautious with nomograms that are currently advocated as tools for selection of patients for adjuvant therapy [23].

In addition to the histological biomarkers investigated in this study, molecular biomarkers such as microsatellite instability (MSI) and *RAS/RAF* mutational status are entering the clinic. However, the implications of these molecular biomarkers in rectal

cancer are as yet undefined. Large cohort studies are warranted as the next step for more personalized treatment in rectal cancer.

In conclusion, in the current study we confirmed that stage independent biomarkers in locally advanced rectal cancer are significantly associated with adverse survival, especially when two or more biomarkers were present. More importantly, these factors do not have predictive value, and do not warrant an indication for adjuvant therapy in rectal cancer patients treated with neoadjuvant short course radiotherapy and TME surgery.

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# PART II

## Tumour immune interactions, HLA-G expression in colorectal cancer

# Chapter 5

## HLA-G and classical HLA class I expression in primary colorectal cancer and associated liver metastases

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# ABSTRACT

De novo expression of HLA-G has been demonstrated in colorectal cancer. HLA-G, amongst others, inhibits natural killer cell function, contributing to host immune defense evasion. Another mechanism to escape anti-tumor immunity is loss of HLA class I. Therefore, we determined HLA-G and HLA class I expression on primary colorectal tumors and associated liver metastases, in order to get insight in the metastasizing process regarding escaping anti-tumor immunity. HLA-G expression was evaluated using three mAbs; 4H84, MEM-G/1 and MEM-G/2. In total 81 colorectal cancer patients were evaluated. Formalin-fixed paraffin-embedded tissue sections of primary tumors and associated liver metastases, were immunohistochemically stained. A concordance between expression or loss/downregulation in the primary tumor and associated liver metastasis regarding HLA class I expression was observed in 80% of the cases. In contrast with the hypothesis of escaping NK cell-killing, we demonstrated for each HLA-G detecting mAbs used in this study, that the majority of the primary tumors that positively stained for HLA-G did not express HLA-G in the associated liver metastasis. Furthermore, we revealed the existence of non-specific binding and in addition we found that the different epitopes of HLA-G detected by 4H84, MEM-G/1 and MEM-G/2 mAbs were expressed differentially in colorectal tumor tissues.

# INTRODUCTION

There is accumulating evidence which supports the notion that the immune system plays an important role in tumorigenesis. Currently, the concept of cancer immune-editing, resulting in an escape of the host defense immunity is widely accepted [1]. Therefore, evasion of immune surveillance is considered one of the emerging hallmarks of cancer [2]. Different mechanisms are used by tumor cells to differentiate towards cells with reduced immunogenicity. Understanding these complex mechanisms is a major challenge. Two important mechanisms in the escape from the host immune recognition and destruction is expression of the non-classical human leukocyte antigen-G (HLA-G) and complete loss or downregulation of classical HLA class I molecules.

HLA-G is rarely expressed in healthy tissues except in immune privileged sites, such as the placenta, where it is involved in the immune tolerance towards the maternal immune system [3, 4]**.** De novo expression of HLA-G has been reported in human malignant cells, including colorectal cancers [5-7]**.** A crucial role for the tumor-driven expression of the HLA-G molecule in escaping the hosts' immune surveillance is by direct interaction with inhibitory receptors on T lymphocytes and peripheral blood natural killer (NK) cells [5], summarized as an immune checkpoint inhibiting antitumor responses. This explains why expression of the HLA-G molecule on cancer cells is associated with higher tumor grade and poor prognosis [8].

A second well-known mechanism to escape anti-tumor immunity is downregulation or total loss of the classical HLA class I proteins. Tumor cells expressing HLA class I present tumor-associated antigens (TAA) on their cell surface and consequently are recognized and destructed by cytotoxic T-cells (CTL) [9]. Therefore, downregulation or total suppression of HLA class I results in inefficient TAA presentation and less recognition by CTL. In approximately 15% up to 75% of colon cancers the phenomena of downregulation or loss is observed [10-12]. Additionally, studies on the prognostic value of diminished expression of HLA class I in colon and rectal cancer patients, showed in general a worse overall survival in patients with loss or downregulation of HLA class I, compared with patients expressing HLA class I in tumor cells [13-15]. On the other hand**,** tumor cells with a complete loss of HLA class I, once they metastasize to the circulation, are targets for elimination by NK cells. Therefore, loss of HLA class I could also be related to a better patient survival. Especially, colorectal cancer patients with a microsatellite instable (MSI) type tumor and complete loss of HLA class I, were associated with a reduction of metastatic disease [16, 17].

In order to get insight in the immunogenic profile of metastasizing cells, we investigated

the expression of the immune-related tumor markers HLA-G and classical HLA class I in primary colorectal cancer and associated liver metastasis by immunohistochemistry. Furthermore, we investigate the correlation between synchronous and metachronous occurrence of the metastasis regarding HLA-G and HLA class I expression.

Immunohistochemistry is a widely accepted technique, although detecting HLA-G expression with immunohistochemistry is still controversial. For example, the reported level of HLA-G expression, detected by immunohistochemistry, in colorectal cancer differs in literature ranging from 20.3% by Zeestraten *et al. to* 72% by Guo *et al.* [15, 18]. These discrepancies are not solely observed in colorectal cancer. For example, HLA-G expression in melanoma cell lines was demonstrated by Paul *et al.,* whereas, no HLA-G expression was observed by Frumento *et al.* [19, 20]. These discrepancies in melanoma cell lines were attributed to the fact that different, not commercially available, monoclonal antibodies (mAbs) were used. Currently, several mAbs are commercially available, but many of these show cross reactivity. For instance the commercially available and widely used 4H84 mAb cross reacts with the ß2-microglubulin (ß2m) free classical HLA class I antigens [21]. For that reason it is recommended to use multiple HLA-G specific mAbs [21, 22]. Therefore, in this study we used three different antibodies to detect HLA-G expression and we compared the reactivity pattern of the different anti-HLA-G mAbs.



## MATERIALS AND METHODS

### PATIENTS AND TISSUE SAMPLES

The study cohort consisted of patients diagnosed with colorectal cancer between 1986 and 2001 who underwent a hepatic resection for metastatic colorectal cancer at the department of Surgery, Leiden University Medical Center. Sufficient formalin-fixed paraffin-embedded tumor tissue, of both primary tumor and associated liver metastasis, was available of 81 patients. Patient and tumor data were retrieved from patient's medical files and pathology reports. Patients were divided in two groups, based on having synchronous or metachronous metastasis. Synchronous metastasis is defined as liver metastases diagnosed before or during the resection of the primary tumor, metachronous metastasis were defined as metastases diagnoses after the resection of the primary tumor [23].

### ANTIBODIES

HLA-G targeting mouse monoclonal antibodies 4H84 (Exbio, Czech Republic), MEM-G/1 (Abcam, ab 7759) and MEM-G/2 (AbCam, ab26090), recognizing all HLA-G

isoforms, were used to assess HLA-G expression on formalin-fixed paraffin-embedded tumor tissue sections. To visualize the classical HLA class I protein, mouse monoclonal antibodies HCA2 and HC10 were used. Both of these antibodies were kindly provided by Prof. Dr. J. Neefjes, NKI Amsterdam, the Netherlands. The mAb HCA2 recognizes all HLA-A chains (except HLA-A24), and some HLA-B, HLA-C, HLA-E, HLA-F and HLA-G chains [24, 25]. The mAb HC10 recognizes HLA class I heavy chains and reacts mostly with HLA-B and HLA-C and some HLA-A (HLA-A10, HLA-A28, HLA-A29, HLA-A30, HLA-A31, HLA-A32, HLA-A33) [26].

### IMMUNOHISTOCHEMISTRY

Paraffin blocks with tissue from the primary colorectal tumors and associated liver metastases were collected. Tissue sections of 4 µm were cut and processed for immunohistochemical staining. Briefly, after deparaffinization and rehydration endogenous peroxidase was blocked by incubating the sections in a 0.3% hydrogen peroxide solution for 20 min. Antigen retrieval was performed as follows; slides for staining with mAbs MEM-G/1, MEM-G/2, HCA2 and HC10, were heated 10 minutes at 95ºC in; pH low Target Retrieval Solution (Dako, Heverlee, Belgium). Slides for staining with mAb 4H84 were heated 10 minutes at 95ºC in; pH high Target Retrieval Solution (Dako, Heverlee, Belgium). The sections were incubated, in pre-determined optimal dilutions, overnight with 4H84 (dilution 1:500), MEM-G/1 (dilution 1:400), MEM-G/2 (dilution 1:400), HCA2 (dilution 1:800) and HC10 (dilution 1:500). Sections were washed three times for 5 minutes in phosphate *buffered* saline (PBS) followed by 30 minutes incubation with EnVision+ System-HRP anti mouse (DAKO, Glostrup, Denmark). The staining was completed by 10 minutes incubation with 3,3’-diaminobenzidine (DAB)+ Substrate-Chromogen System (DAKO, Glostrup, Denmark). Finally, sections were washed two times in demineralised water, counterstained with haematoxylin, dehydrated and mounted in Pertex.

Nonspecific protein binding was blocked with 5% goat serum before incubation with MEM-G/1 and MEM-G/2 for making a comparison between blocking and not using a block.

Placenta tissue served as an external positive control for staining with anti-HLA-G mAbs. Human tonsil tissue served as an external positive control for classical HLA class I staining. Normal epithelium, stromal cells or lymphoid cells served as an internal positive control for the HCA2 and HC10 antibodies. Negative controls consisted of slides that underwent the whole immunohistochemistry protocol without the use of the primary antibody.

### EVALUATION OF IMMUNOHISTOCHEMISTRY

Microscopic analysis was performed by two independent observers in a blinded manner (M.S and M.H.K). The Cohen's Kappa coefficient ranged from 0.64 to 1.00 for al staining's, which indicates a substantial agreement between the observers.

Three different mAbs were used to evaluate HLA-G expression (4H84, MEM-G/1 and MEM-G/2) and the presence or absence of positive stained tumor cells was determined. The concordance between expression in the primary tumors and associated liver metastases was evaluated for each mAb separately.

For HCA2 and HC10 the percentage of tumor cells with a positive stained membrane were assessed. According to the International HLA and Immunogenetics Workshop [27], the HLA class I expression status was determined as follows; HLA class I expression is defined as 5% or more of the tumor cells stained positive for HCA2 and HC10. Loss of HLA class I expression is defined as less than 5% of the tumor cells stained positive for both HCA2 and HC10 and downregulation of HLA class I is defined as less than 5% of tumor cells expressing either of the markers.



### STATISTICAL ANALYSIS

Statistical analyses were performed using the statistical package SPSS (version 20.0 for windows; SPSS Inc.). The Chi-square test and Cohen's Kappa coefficient were used to perform the statistical analysis.

## RESULTS

### PATIENTS

Tissue was collected from 81 patients with primary colorectal cancer and associated liver metastasis. Patient characteristics are shown in Table I. Of the 81 patients 38% (31/81) were diagnosed with synchronous metastases and 62% (50/81) were diagnosed with metachronous metastases.

### EVALUATION OF THE REACTIVITY OF 4H84, MEM-G/1 AND MEM-G/2

To evaluate the reactivity of the different HLA-G antibodies used in tissue sections of colorectal tumors and liver metastases, we first investigated the effect of blocking with 5% goat serum. Figure 1 shows the different staining patterns for MEM-G/1 with and without the use of 5% goat serum. As shown in Figure 1A, a MEM-G/1 staining, positive stained tumor cells were observed without the use of 5% goat serum and a completely

negative tumor cells were observed with the use of a 5% goat serum block, revealing the existence of non-specific binding in colorectal tumor tissue. As shown in Figure 1B, in some cases positive stained tumor cells remain positive despite the use of a 5% goat serum. Correspondingly, for MEM-G/2 non-specific binding was observed in a same way as MEM-G/1 (data not shown). In contrast to MEM-G/1 and MEM-G/2 the staining pattern with 4H84 was not influenced by blocking with 5% goat serum (data not shown).

In contrast, as shown in Supplementary figure 1, all anti-HLA-G mAbs showed a similar reactivity pattern in placenta tissue. Blocking with 5% goat serum did not affect this staining pattern, revealing the specificity of these HLA-G antibodies in placenta.

**Table 1.** Patient characteristics of total patients population.

| | n = 81 | % |
|---|---|---|
| Sex | | |
| Male | 53 | (65) |
| Female | 28 | (35) |
| Age median (years) | 59.0 | (±8.8) |
| Stage | | |
| I | 3 | (4) |
| II | 6 | (7) |
| III | 41 | (51) |
| IV | 31 | (38) |
| Differentiation | | |
| Good | 4 | (5) |
| Moderate | 66 | (82) |
| Poor | 10 | (12) |
| Missing | 1 | (1) |
| Location | | |
| Colon | 43 | (53) |
| Rectosigmoid | 9 | (11) |
| Rectum | 29 | (36) |
| Metastasis | | |
| Synchronous | 31 | (38) |
| Metachronous | 50 | (62) |



Representative images of HLA-G staining with the three different HLA-G mAbs in sequential tissue sections of colorectal tumor tissue are shown in Figure 2. As demonstrated in this figure different staining patterns, regarding positive and negative stained tumor cells, were observed among the three different HLA-G mAbs. The colorectal tumor tissue in panel A stained negative for 4H84 and MEM-G/1, although it was positive for staining with MEM-G/2. The sequential tissue sections in panel B show tumor cells stained positive for 4H84 and negative for MEM-G/1 and MEM-G/2. The

sequential tumor tissue sections in panel C were positive stained for 4H84, MEM-G/1 and MEM-G/2 in the corresponding tumor cells. This suggests that the different epitopes of HLA-G detected by these mAbs are expressed differentially in colorectal tumor tissues.

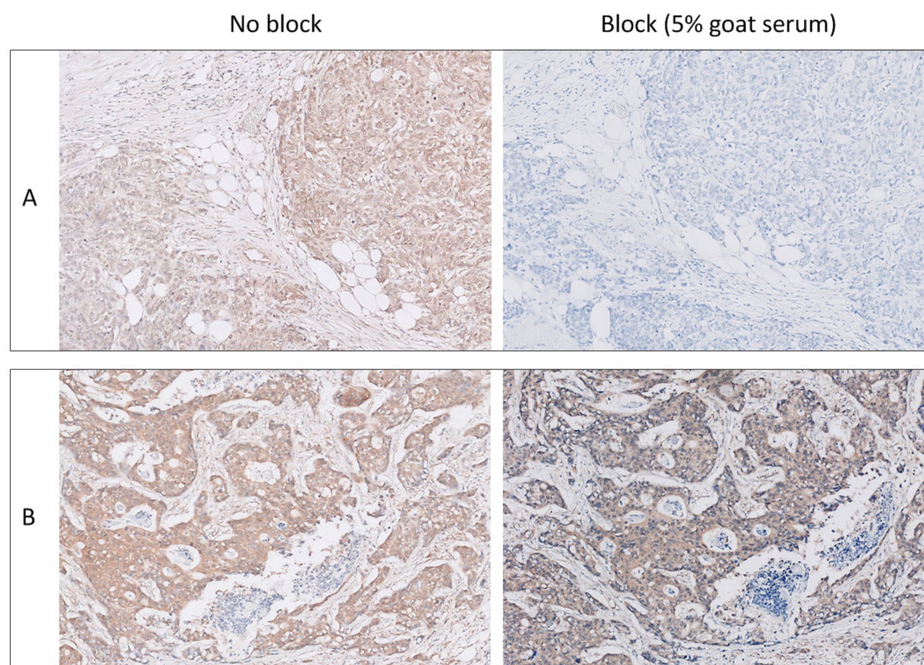


**Figure 1.** Representative images of MEM-G/1 staining with and without the use of 5% goat serum. **A.** In the left panel MEM-G/1 positive stained colorectal tumor section without the use of 5% goat serum. In the right panel a sequential tissue section negatively stained with MEM-G/1, with the use of 5% goat serum. **B.** In the left panel MEM-G/1 positive stained colorectal tumor section without the use of 5% goat serum. In the right panel a sequential tissue section also positive stained with MEM-G/1, with the use of 5% goat serum.

## HLA-G AND HLA CLASS I EXPRESSION IN PRIMARY TUMOR AND ASSOCIATED LIVER METASTASIS

HLA-G expression was analyzed for each antibody separately, as a consequence of the previously suggested differentially expressed epitopes of HLA-G in colorectal cancer tissues detected by 4H84, MEM-G/1 and MEM-G/2 mAbs. For the analysis of the results data from tissues sections blocked with 5% goat serum were used, in order to avoid inclusion of false positive stained tissue sections. Due to loss of material during the staining procedure the total number of tissue sections evaluated is not reaching 81.

Positive staining for HLA-G in the primary tumor was observed in 29% for staining with 4H84, 6% for staining with MEM-G/1 and 10% for staining with MEM-G/2 (Table 2). In the liver metastases 30%, 4% and 0% showed positivity for staining with 4H84, MEM-G/1 and MEM-G/2 respectively (Table 2). The analysis for primary tumor and associated liver metastasis sets included 77, 76 and 76 sets for 4H84, MEM-G/1 and MEM-G/2, respectively (Table 3).

**Table 2.** Frequencies of 4H84, MEMG/1 and MEM-G/2 positive and negative stained tumor cells in tissue sections of primary colorectal cancer and liver metastases. For MEMG/1 and MEM-G/2 the effect of blocking with 5% goat serum are shown. Due to loss of material during the staining procedure the total number of tissue sections evaluated is not reaching 81.

| | **Colorectal tumor** | | **Liver metastasis** | |
|---|---|---|---|---|
| | Positive | Negative | Positive | Negative |
| 4H84 | 23 (29%) | 57 (71%) | 23 (30%) | 55 (71%) |
| MEM-G\1 | | | | |
| Block – | 28 (35%) | 53 (65%) | 10 (13%) | 68 (87%) |
| Block + | 5 (6%) | 74 (94%) | 3 (4%) | 75 (96%) |
| MEM-G\2 | | | | |
| Block – | 15 (19%) | 66 (82%) | 0 (0%) | 78 (100%) |
| Block + | 8 (10%) | 71 (90%) | 0 (0%) | 78 (100%) |

A concordance between expression or no expression in the primary tumor and associated liver metastasis was observed in 77% regarding staining with 4H84. In addition, 9 out of 22 of the primary tumors were positively stained for 4H84 and negatively stained in the associated liver metastasis, which accounts for 12% of the primary tumor and liver metastases sets. For MEM-G/1, in 91% of the primary tumor and associated liver metastasis sets, no HLA-G expression was detected in either of the tissues. Furthermore, 5% of the primary tumor and liver metastasis sets were positive for MEM-G/1 in the primary tumor and negative for MEM-G/1 in the liver metastasis. Compared to MEM-G/1, a similar pattern was observed for the staining with MEM-G/2, all primary tumors that stained positive for MEM-G/2 were negative in the associated liver metastases. Furthermore, no significant differences were observed between HLA-G expression in primary tumor regarding synchronous or metachronous onset of metastases (4H84 $p$=0.140, MEM-G/1 $p$=0.633, MEM-G/2 $p$=0.139).

The analysis of HLA class I expression included 80 primary tumors en 77 associated liver metastasis. Due to loss of material during the staining procedure the results of 5 sets of primary tumor and associated liver metastasis could not be analyzed. Representative images of HLA class I expression and loss of expression are shown in Figure 3. The

majority of the primary tumors (72%) and liver metastases (64%) were expressing HLA class I. A concordance between the expression or loss/downregulation in the primary tumor and associated liver metastasis regarding HLA class I expression was observed in 80% of the cases (Table 3). Furthermore, no significant difference ($p$=0.698) was observed between the HLA class I expression profiles and the synchronous or metachronous onset of liver metastases.

**Table 3.** Cross tables comparing expression of HLA class I and HLA-G in primary colorectal tumors (primary CRC) and associated liver metastases. For each mAbs detecting HLA-G (4H84, MEM-G/1 and MEM-G2) a separate cross table is shown. For MEM-G/1 and MEM-G/2 cross tables with and without blocking with 5% goat serum are shown.

| | Liver metastases | |
|---|---|---|
| **HLA Class I** | Expression | Loss + Downregulation |
| **Primary CRC** | | |
| Expression | 44 (58%) | 11 (15%) |
| Loss + Downregulation | 4 (5%) | 17 (22%) |

| | Liver metastases | |
|---|---|---|
| **4H84** | Positive | Negative |
| **Primary CRC** | | |
| Positive | 13 (17%) | 9 (12%) |
| Negative | 9 (12%) | 46 (60%) |

| | Liver metastases | |
|---|---|---|
| **MEM-G/1** | Positive | Negative |
| **Primary CRC** | | |
| Positive | 1 (1%) | 4 (5%) |
| Negative | 2 (3%) | 69 (91%) |

| | Liver metastases | |
|---|---|---|
| **MEM-G/2** | Positive | Negative |
| **Primary CRC** | | |
| Positive | 0 (0%) | 8 (11%) |
| Negative | 0 (0%) | 68 (90%) |

| | Liver metastases | |
|---|---|---|
| **MEM-G/1 (No block)** | Positive | Negative |
| **Primary CRC** | | |
| Positive | 6 (8%) | 21 (27%) |
| Negative | 4 (5%) | 47 (60%) |

| | Liver metastases | |
|---|---|---|
| **MEM-G/2 (No block)** | Positive | Negative |
| **Primary CRC** | | |
| Positive | 0 (0%) | 15 (19%) |
| Negative | 0 (0%) | 63 (81%) |

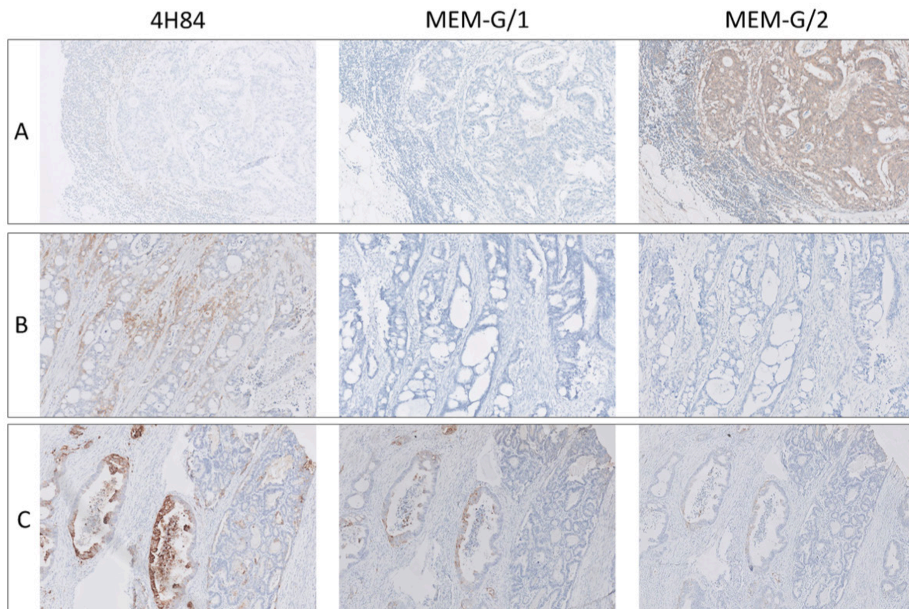


**Figure 2.** Different reactivity patterns among the three mAbs (4H84, MEM-G/1 and MEM-G/2) in sequential tissue sections of colorectal tumors. For the staining with MEM-G/1 and MEM-G/2 blocking with 5% goat serum was used.

**A.** Sequentially sections of colorectal tumor tissue. Negative stained for 4H84 and MEM-G/1. Positive stained for MEM-G/2. **B.** Sequentially sections of colorectal tumor tissue. Positive stained for 4H84 and negative stained for MEM-G/1 and MEM-G/2. **C.** Three sequentially tissue sections positive stained for 4H84, MEM-G/1 and MEM-G/2.

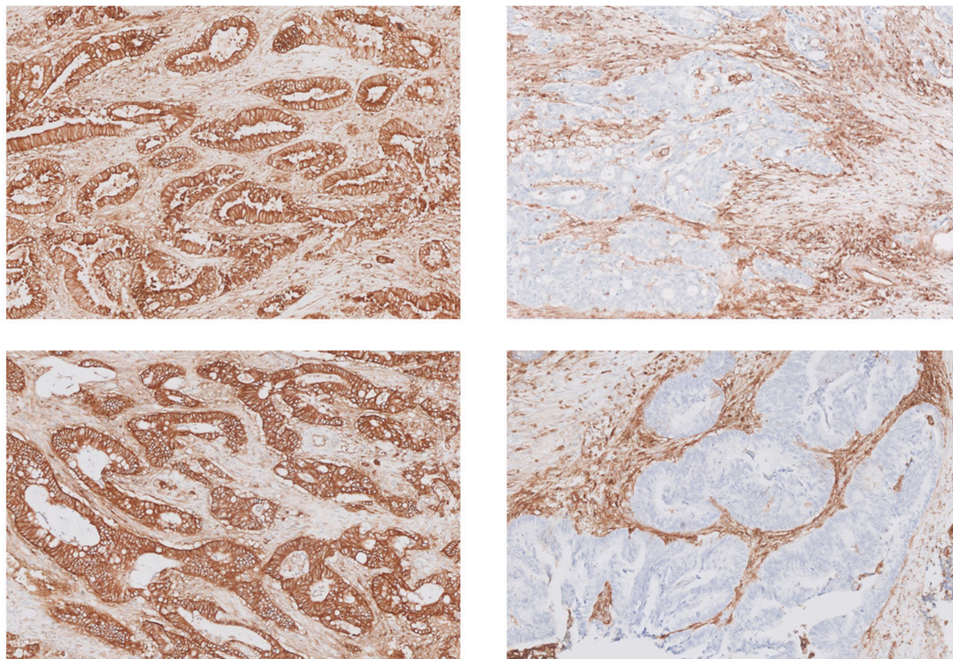

**Figure 3.** Representative images of immunohistochemical HLA class I staining. HLA class I expression is defined as 5% or more of the tumor cells stained positive for HCA2 and HC10. Loss of HLA class I expression is defined as less than 5% of the tumor cells stained positive for both HCA2 and HC10. Downregulation of HLA class I is defined as less than 5% of tumor cells expressing either of the markers. **A.** HCA2 positive tumor **B.** HCA2 negative tumor **C.** HC10 positive tumor **D.** HC10 negative tumor

## DISCUSSION

HLA-G is thought to act as an immune checkpoint molecule; de novo expression of HLA-G on tumor cells results in activation of immune response inhibitory signalling. Therefore, HLA-G is considered as a potential target for optimization of current cancer immunotherapy strategies [28]. In previous research no significant differences in overall survival associated with HLA-G expression on tumor cells was observed in patients with colon and rectal cancer [13, 15]. In contrast, Guo *et al* did show a worse patient survival regarding HLA-G expression [18]. In our study, the included patients were all experiencing metastatic disease and their clinical condition allowed resection of their liver metastases. Therefore, it was not possible to calculate survival outcomes. We were able to perform analyses regarding synchronous or metachronous onset of liver

metastases. No significant difference between synchronous or metachronous onset of liver metastasis was observed regarding HLA-G expression.

Previous studies showed a worse survival rate in patients not expressing HLA class I on the cell surface of their tumors. Furthermore, an even more unfavorable prognosis was observed when combining HLA class I downregulation with HLA-G expression in colorectal cancer patients, which supports the hypothesis of an immune escape advantage for tumor cells with diminished HLA class I expression [13-15]. In contrast, we found that the majority of the tumor cells within the liver metastases did express HLA class I. Therefore, HLA class I loss may be an advantage in the hosts immune defense evasion, but not absolutely required for formation of metastases.

The precise immune phenotype of metastasizing cells is difficult to reveal, due to the heterogeneity of tumors. Studying only one immune marker is not sufficient to understand the complete immune escape mechanism. Immune markers are related to each other and combining markers could give more insight in the complex mechanism of evasion of the host's immune surveillance. As hypothesized, cells lacking HLA class I protein expression on their cell surface are a NK cell target in the circulation. Besides, cells expressing HLA-G are supposed not to be lysed by NK cells in the circulation. In contrast with the hypothesis of escaping lysis by NK cells, we demonstrated for each HLA-G detecting mAbs used in this study, that the majority of the primary tumors positive stained for HLA-G do not express HLA-G in the associated liver metastasis. This suggests that HLA-G is not a major contributor to the metastatic process in the circulation. It would have been interesting to combine HLA-G expression and HLA class I loss. Nevertheless, our data suggest that the different epitopes of HLA-G detected by 4H84, MEM-G/1 and MEM-G/2 are expressed differentially in colorectal tumor tissues. The 4H84 mAb is a widely used mAb to detect HLA-G. However, cross reactivity with ß2m free classical HLA class I molecules on activated leukocytes has been demonstrated [21]. This could results in false recognition of HLA-G expression in pathologies that are recognized by leucocyte infiltration such as colorectal cancer. Consequently, it is recommended not to rely on an analysis with solely 4H84 but to detect HLA-G with a number of different mAbs [21, 22]. Accordingly, we decided to stain the tissues with three different mAbs targeting HLA-G. It should be noted that to the best of our knowledge a consensus about interpreting the results of different antibodies is not available. Furthermore, we revealed the existence of non-specific binding and in addition our data suggest that the different epitopes of HLA-G detected by 4H84, MEM-G/1 and MEM-G/2 mAbs are expressed differentially in colorectal tumor tissues. However we did reveal the specificity of these HLA-G antibodies in placenta.

In literature, the reported level of HLA-G expression, detected by immunohistochemistry, in colorectal cancer and other cancer types differs extensively [15, 18, 22]. Besides the variation in reported HLA-G expression, the proportion of positive stained cells in a tumor is highly variable as well [22]. We suggested that the different epitopes of HLA-G detected by 4H84, MEM-G/1 and MEM-G/2 mAbs are expressed differentially in colorectal tumor tissues. Additionally, HLA-G might be expressed differently in specific tumors or perhaps some HLA-G isoforms might be more prominent in some specific tumor types. G*enomic instability* is a characteristic of almost all human cancers causing mutations in the HLA-G gene, which could be an explanation for the different staining patterns.

Based on the results of this study, we have to conclude that it is not possible to compare the patterns of reactivity or combine the results to one "HLA-G expression value" due to the impressive different patterns observed with the antibodies used. For that reason, future addition of biochemical analyses will be necessary to evaluate the binding patterns of the three HLA-G mAbs. However, in the current study this was not feasible as our analyses were performed on paraffin-embedded tissue sections obtained from the pathology achieve and only a limited amount of tumor tissue from these patients was available. This might be *considered as a limitation of our study.*

We have to realize that immunohistochemistry for HLA-G is extremely difficult to interpret. In line with the results in this study, conclusions based on immunohistochemistry have to be drawn very carefully. Especially literature based on one anti-HLA-G mAb should be interpreted with caution as the noted cross-reactivity and existence of non-specific binding may lead to an over-estimation of HLA-G expression in cancer.

In conclusion, the non-classical HLA class I molecule HLA-G is an interesting and promising protein in cancer research and is considered as an attractive candidate molecule for therapeutic intervention. However, an emerging need for standardization of the procedures to detect HLA-G, especially in paraffin sections, is warranted. It is therefore crucial to completely unravel their binding domains and cross reactivity patterns. Until then, it is difficult to compare different staining patterns and draw conclusions related to HLA-G expression in cancer pathologies.

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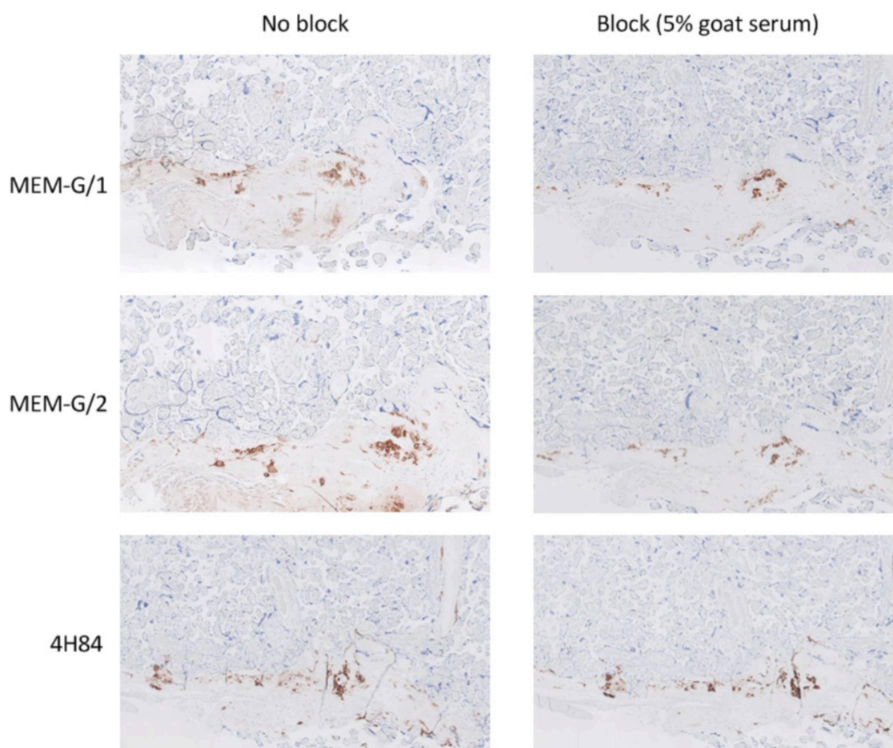
# SUPPLEMENTARY FILE




**Supplementary figure 1.** Images of sequential tissue sections of placenta tissue, stained with MEM-G/1, MEM-G/2 and 4H84 with and without the use of 5% goat serum. As shown all three mAbs have a same reactivity pattern in placenta, in the presence of blocking serum (5% goat serum).

# Chapter 6

## Promoter methylation and mRNA expression of *HLA-G* in relation to HLA-G protein expression in colorectal cancer

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## ABSTRACT

Expression of human leukocyte antigen-G (HLA-G) is a suggested mechanism used by tumor cells to escape from host immune recognition and destruction. Advances in the field have made it evident that HLA-G is expressed in different types of malignancies including colorectal cancer (CRC). We analyzed HLA-G expression in 21 low passage CRC cell lines. The level of DNA methylation of the *HLA-G* gene and the presence of mRNA encoding HLA-G was measured. Moreover, HLA-G protein expression was determined by flow cytometry and immunohistochemistry (IHC). IHC was performed with three different monoclonal antibodies (mAbs) (4H84, MEM-G/1 and MEM-G/2). In addition, HLA-G protein expression was measured in matching primary tumor tissues. RNA analysis using RT-PCR followed by sequencing in 6 samples indicated strong homology of the PCR product with HLA-G3 in 5 samples. In accordance, in none of the cell lines, HLA-G1 expression was detected by flow-cytometry. Furthermore, no association between *HLA-G* DNA methylation patterns and *HLA-G* mRNA expression was observed. In addition, different immunohistochemical staining profiles among various anti-HLA-G mAbs were observed. In conclusion, the results of this study show that the HLA-G3 isoform was expressed in some of the CRC cell lines irrespective of the level of DNA methylation of *HLA-G*.

# INTRODUCTION

Evasion of immune surveillance is considered one of the emerging hallmarks of cancer [1, 2]. Understanding the complex mechanisms used by tumor cells to differentiate towards cells with reduced immunogenicity is a major challenge. It is suggested that tumor cells can escape immune recognition and destruction by expression of human leukocyte antigen-G (HLA-G) and it provides to an explanation why expression of the HLA-G molecule is associated with poor patient prognosis [3-5]. HLA-G is a non-classical HLA class I (class Ib) molecule, expressed at immune-privileged sites such as the placenta, in extravillous trophoblast cells [6-8]. Alternative splicing of the primary transcript has been reported to result in seven different HLA-G protein isoforms; four membrane-bound (HLA-G1, G2, G3, G4) and three soluble (HLA-G5, G6, G7) isoforms [9]. Advances in the field have made it evident that HLA-G is also expressed in different types of malignancies in a *de novo* manner [10]**.** This tumor driven expression of HLA-G inhibits the function of several types of immune cells, among which T cells and natural killer (NK) cells, inhibits proliferation of immune cells, and can additionally induce expansion of an immunosuppressive T cell subset [11]. Therefore HLA-G expression in tumors was recently described as an immune checkpoint molecule [12]**.** Expression of HLA-G appears as a promising clinical prognostic factor in several types of cancer, including colorectal cancer (CRC) [13-16]. However, among and within different tumors types variances in HLA-G expression were observed [17]. For example, HLA-G expression has been reported by Zeestraten *et al.* in 20% of colon tumors, whereas Guo *et al.* reported levels up to 72% in CRC [15, 18]. Immunohistochemistry (IHC) is a widely accepted technique to detect HLA-G expression, although the obtained results are still controversial [17, 19]. It is important to note that most studies used a single type of monoclonal antibody (mAb), usually 4H84. However, in CRC we showed previously discrepant expression profiles among various types anti-HLA-G mAbs [20]. For that reason and because of the known cross reactivity of mAb 4H84, it is recommended to use multiple HLA-G specific mAbs [17, 19]. Furthermore, additional molecular biological and biochemical analyses will be required to evaluate HLA-G expression in cancer and to firmly validate HLA-G expression patterns.

Previously it has been shown that HLA-G transcription is regulated by epigenetic mechanisms, including by DNA methylation [21, 22]. Nevertheless, many of the established cell lines utilized in research have been in culture for decades and may present aberrant genetic and epigenetic characteristics. In the current study we therefore investigated *HLA-G* DNA methylation level in 21 recently established CRC cell lines never investigated before for HLA-G expression. Furthermore, the presence of HLA-G mRNA was measured. Membrane expression of the HLA-G protein was evaluated by flow cytometry and IHC. We used three different anti-HLA-G mAbs for analyzing

expression of HLA-G by IHC in the CRC cell lines and results were compared with paraffin-embedded tumor tissue of which the tumor cell lines were derived from.

# MATERIALS AND METHODS

## TUMOR CELL LINES

21 CRC cell lines were established from primary CRC tumors and colorectal liver metastasis at the Department of Pathology, LUMC (Table 1). The cell lines have been extensively characterized for several cancer related mutations by Boot et al. [23]. The CRC cell lines were cultured in RPMI-1640 (Gibco™,Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal calf serum (FCS). Dulbecco's Modified Eagle medium (DMEM)/F12 (Gibco™) with 10% FCS was used for culturing JVE222 and JVE371. DMEM (Gibco™) with 10% FCS was used to culture the choriocarcinoma cell line JEG-3, known for expressing all HLA-G isoforms.



**Table 1.** Characteristic of the colorectal cancer cell lines. Cell line name, passage number of *in vitro* culture at RNA isolation, and tumor location and morphology.

| Cell Line | Passage | Location | Tumor morphology |
|---|---|---|---|
| JVE015 | p.15 | Sigmoid | Unknown |
| JVE017 | p.13 | Cecum | Unknown |
| JVE044 | p.13 | Rectum | Unknown |
| JVE059 | p.15 | Colon | Adenocarcinoma |
| JVE103 | p.13 | Liver[1] | Adenocarcinoma |
| JVE109 | p.21 | Colon | Adenocarcinoma |
| JVE114 | p.13 | Liver[1] | Adenocarcinoma |
| JVE127 | p.12 | Colon | Mucinous adenocarcinoma |
| JVE187 | p.13 | Liver[1] | Adenocarcinoma |
| JVE192 | p.13 | Colon | Mucinous adenocarcinoma |
| JVE207 | p.14 | Colon | Adenocarcinoma |
| JVE222 | p.19 | Colon | Adenocarcinoma |
| JVE 241 | p.8 | Cecum | Mucinous adenocarcinoma |
| JVE253 | p.6 | Liver[1] | Mucinous adenocarcinoma |
| JVE367 | p.7 | Ileocecal junction | LNEC[2] |
| JVE371 | p.7 | Liver[1] | Adenocarcinoma |
| JVE528 | p.8 | Colon | Adenocarcinoma |
| JVE774 | p.17 | Rectum | Adenocarcinoma |
| KP283T | p.6 | Liver[1] | Adenocarcinoma |
| KP363T | p.8 | Colon | Adenocarcinoma |
| KP7038T | p.7 | Colon | Adenocarcinoma |

### DNA PROMOTER METHYLATION

DNA isolation was performed with the Wizard genomic DNA purification kit (Promega, Madison, WI,USA) according to the manufacturer's instructions. Bisulfite conversion was performed with EZ DNA Methylation Gold kit (Zymo Research Corp, Orange, CA, USA) on genomic DNA, according to the manufacturer's instructions, using an input of 200ng of DNA. Primers (Biolegio, Nijmegen, the Netherlands) previously designed by Holling *et al.* [21], targeting the minimal promoter and part of CpG island-3 in exon 3, were used with an additional M13-tail (underlined) for sequencing purposes. BSHLA-GF1:*5'TGTAAAACGACGGCCAGTGATTTAGGGAGATATTGAGATAGAA-3'* and BSHLA-GR1:*5'CAGGAAACAGCTATGACCCACCTAATAAAAATAAAAACTAAAACC-3'* detecting the minimal promoter. BSHLA-GF3: *5'TGTAAAACGACGGCCAGTATATTTTTTAGTGGATGATTGGTTG-3'* and BSHLA-GR3: *5'CAGGAAACAGCTATGACCCCTCCAAATAAACTCTCCTTTATTC-3'*spanning a part of exon 3. PCR was performed in a final volume of 10 µl using AmpliTaq Gold polymerase (Thermo Fisher Scientific, Inc., Waltham, MA, USA) (1 unit per reaction), 1X AmpliTaq Gold buffer, $MgCl_2$ (1.9mM), dNTPs (200µM each), Syto9 (0.4µM) and pooled primers (2.5µM) according to the manufacturer's instructions with 100 ng of genomic DNA of each of the 21 novel CRC cell lines. The following PCR protocol was executed: 5min 95°C, 15x (15sec 95°C, 30sec 59°C,with 0.6°C decrease every cycle, 20sec 72°C), 39x (10sec 95°C, 30sec 62°C, 20sec 72°C), 1min 60°C, 10sec 65°C, 5sec 95°C). The PCR product was analyzed using QIAxcel (QIAGEN, Hilden, Germany) followed by product purification with purifying plates (Thermo Fisher Scientific). Samples were sent for sequencing to Macrogen, Amsterdam, the Netherlands. In order to analyze CpG nucleotide methylation the Epigenetic Sequencing Methylation Software (ESME) was used. Methylation patterns were deduced from aligning the bisulfite-converted sequences of the CRC cell lines to the genomic sequence of the *HLA-G* gene (NG_029039.1). DNA methylation levels were quantified by calculation of the total methylation percentage of the CpG sites. The total methylation percentage was calculated as the sum of methylated CpG sites divided by the number of CpG sites. The methylation pattern of JEG-3 was used as a comparative parameter, not as a reference parameter.



### RNA ISOLATION, cDNA SYNTHESIS

Trypsin/EDTA was used to harvest cultured JEG-3 cells for RNA isolation with the RNeasy mini-kit (QIAGEN, Limburg, Netherlands). DNase treatment with RNase-free DNase Set (QIAGEN) was performed according to the manufacturer's instructions. RNA (1µg) was converted to cDNA using iSCRIPT cDNA synthesis kit (Biorad, Hercules, Califrnia, USA). TRIzol Reagent (Life Technologies, Bleiswijk, the Netherlands) was used to isolate RNA from the 21 CRC cell lines. DNAse treatment was performed in suspension using rDNAse (Macherey Nagel GmbH & Co. KG, Düren, Germany). cDNA was synthesized using 1-2µg RNA, 100ng oligo-dT, 1mM dNTP's, 5U AMV-RT transcriptase and 10U

RNasin (#M5108 and # N2615)(Promega).

### PCR, GEL ELECTROPHORESIS AND SEQUENCING

To detect the different HLA-G isoforms, HLA-G primers (Biolegio) G.257 (exon 2; 5'-'GGAAGAGGAGACACGGAACA-3') and G.1225 (3'-UT; 5'-TGAGACAGAGACGGAGACAT-3') were used, first described by Kirszenbaum *et al.* [24, 25]. For the RT-PCR, 2µL cDNA was used in combination with 1pmol primers in a total volume of 20 µL, containing 1x IQ SYBR Green Supermix (Biorad). The RT-PCR was performed using a CFX96 TOUCH™ (Biorad). The following PCR protocol was executed: 5min 95°C, 49x (30sec 95°C , 30sec 62°C, 1min 72°C ). The final amplified PCR products were run on 1.5% agarose gel. The housekeeping gene GAPDH was used as an positive control [26]. The RT-PCR was performed multiple times. After gel electrophoresis the amplicons were extracted and column purified with a MinElute® PCR Purification kit (Qiagen). The PCR protocol was performed for a second time on the purified amplicons in order to increase the amplicon concentration, needed for sequencing. After column purification the samples were sent to the Leiden Genome Technology Center, LGTC (Leiden, the Netherlands) for Sanger sequencing. Alignment of the obtained sequences was performed using the BLAT-the BLAST-like alignment tool [27]. In addition, sequence alignments with HLA-G3 mRNA (ENST00000376815*)* was performed using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) software [28].



### FLOW CYTOMETRY

The HLA-G1 targeting mAb MEM-G/9 (Exbio, Czech Republic) was used to assess cell surface HLA-G expression in the cell lines. The HLA class I targeting mAb W6/32 (Thermo Fisher Scientific) was used as a positive control. Controls, using the secondary antibodies only, were included for each cell line. Cells were thawed and washed in phosphate-*buffered* saline (PBS)/0.5% BSA twice before and after antibody incubations. Primary antibodies MEM-G/9, control antibody W6/32 and the secondary antibodies IgG1-FITC (PickCell Laboratories, the Netherlands) or IgG2a-FITC (PickCell) were incubated for 30 min on ice. IgG1-FITC and IgG2a-FITC are detecting MEM-G/9 and W6/32 respectively. All antibodies were used at predetermined optimal dilutions. The results were analyzed with FlowJo (Tree Star Inc, OR. USA), to compare the reactivity of targeting antibodies with the conjugated controls.

### IMMUNOHISTOCHEMISTRY

HLA-G targeting mouse mAb 4H84 (Exbio, Czech Republic), MEM-G/1 (Abcam, Cambridge, UK, ab 7759) and MEM-G/2 (AbCam, ab26090), were used to assess HLA-G expression on formalin-fixed paraffin-embedded tissue sections. Cells from the CRC cell lines, were collected by careful scraping from the cell culture flasks, followed by

centrifugation. Conform standard procedures the collected cells underwent formalin fixation and were embedded in paraffin. Paraffin blocks with tumor tissue from which the cell lines were derived, were collected from the pathology archive. Tissue sections of 4 μm were cut from the paraffin blocks and processed for IHC. A standard IHC protocol using the Dako Envision+ (Dako, Glostrup, Denmark) was used. Briefly, after deparaffinization and rehydration endogenous peroxidase was blocked by incubating the sections in a 0.3% hydrogen peroxide solution for 20min (30% hydrogen peroxide, 100x diluted in water). Subsequently, antigen retrieval was performed as follows; slides for staining with mAbs MEM-G/1, MEM-G/2, were heated 10min at 95°C in pH low Target Retrieval Solution (Dako). Slides for staining with mAb 4H84 were heated 10min at 95°C in pH high Target Retrieval Solution (Dako). Sections were blocked with goat serum (5%) in PBS before incubation with MEM-G/1 and MEM-G/2. The sections were incubated, at pre-determined optimal dilutions, overnight with 4H84, MEM-G/1 or MEM-G/2. Next, sections were washed three times for 5min in PBS followed by 30min incubation with EnVision+ System-HRP anti mouse (Dako). The staining was completed by 10min incubation with 3,3'-diaminobenzidine (DAB)+ Substrate-Chromogen System (Dako). Finally, sections were washed twice in demineralized water, counterstained with haematoxylin, dehydrated and mounted in Pertex. Placenta tissue was used as a positive control for all three anti-HLA-G mAbs. Sections serving as negative controls underwent the entire protocol except primary antibody incubations. HLA-G expression was evaluated as positive or negative.



# RESULTS

## HLA-G PROMOTER METHYLATION

Transcription of *HLA-G* may be controlled by epigenetic mechanisms including DNA methylation**.** The methylation levels of 10 CpG dinucleotides in the minimal promoter and 15 CpG dinucleotides in CpG island-3 of *HLA-G* were analyzed in the early CRC cell lines**.** The methylation status was compared with the HLA-G expressing cell line JEG-3. The calculated JEG-3 methylation levels were 72% and 85% in the investigated CpG dinucleotides in the promoter and CpG Island-3, respectively. Compared with JEG-3, lower methylation levels in the minimal promoter were observed in the CRC cell lines JVE103, JVE192 and JVE371. In the CpG island-3 JVE059, JVE114, JVE192, JVE207, JVE222, JVE241, JVE253 JVE371, JVE774 KP283T, and KP7038T showed lower methylation levels compared with JEG-3 (Figure 1). Moreover, occasional cell lines were almost completely methylated in these locations e.g. JVE015 with 99% methylation in the minimal promoter and 96% in the CpG island-3 and JVE187 with 100% methylation

in the minimal promoter and 96% in the CpG island-3. In summary, high variance in methylation level was observed in the cell lines.

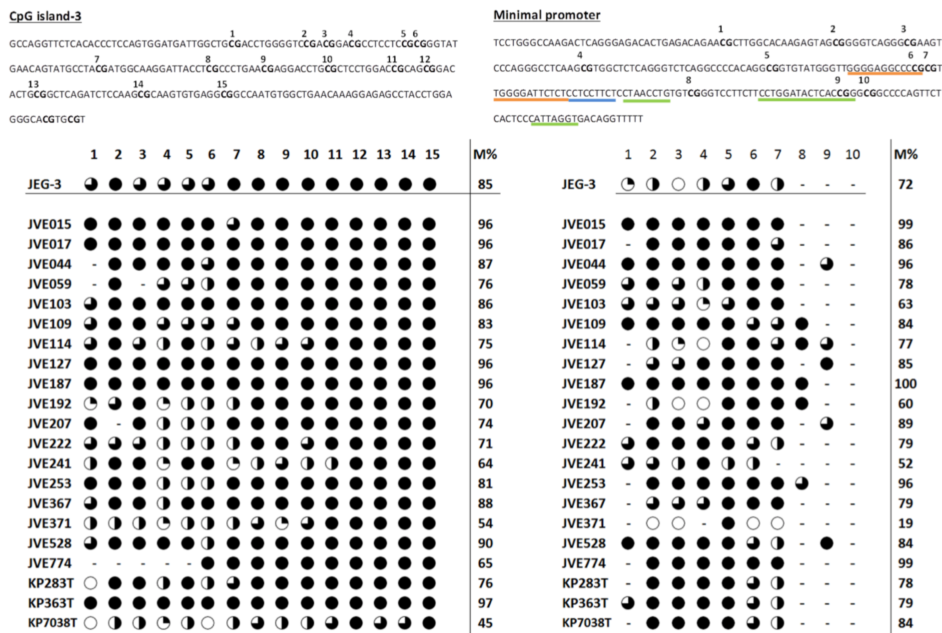


**Figure 1: Methylation percentage for the minimal promoter and CpG island 3 in the 21 colorectal cancer cell lines and JEG-3.** CpG nucleotides in the minimal promoter (1-10) and in the CpG island 3 (1-15). Regions in the minimal promoter resembling the Enh A (orange), ISRE (blue), S, X1, X2 and Y (green) are underlined. Circles indicate percentage of methylation in quartiles. M% reflects the percentage of methylation. Several of the later CpG dinucleotides in the minimal promoter could not be determined due to polymerase slippage in T rich areas. Therefore, an analysis of the reversed sequence was not possible.

## mRNA TRANSCRIPTION OF HLA-G

The presence of HLA-G isoform transcripts was investigated by real time polymerase chain reaction (RT-PCR) of the respective cDNAs followed by Sanger sequencing. The sizes of visible amplicons detected with gel electrophoresis were compared to those obtained with the positive control JEG-3. The results of these analyses showed single amplicons, corresponding in size with detected amplicons in JEG-3, mainly HLA-G3. However, smaller unknown product bands were detected as well (Figure 2A). Furthermore, variability in presence of positive amplicons in the CRC cell lines was

observed among the RT-PCR performed. JEG-3 was positive in each RT-PCR performed and displayed amplicons comparable in size to isoform G5, G1, G6, G2/G4 and G3 (Figure 2A). The positive control GAPDH yielded a product in each PCR performed (Figure 2B). As a consequence of low amplicon concentration it was not possible to sequence each positive sample. In total, 6 purified amplicons were sequenced (Table 2).

**Table 2. HLA-G mRNA expression per cell line.** The table shows the cell lines positive after performing the RT-PCR and DNA sequencing. Due to low amplicon concentration the results obtained by Sanger sequencing of the visible band in cell line JVE187 showed very low signal peaks. Consequently, the obtained sequence was not reliable and is indicated as "unknown". Cell line JVE371 does show homology with HLA-G, however it was not possible to determine the isoform and is therefore indicated as "unknown".

| Cell Line | Positive PCR[1] | Sequence corresponds to HLA-G[2] | HLA-G isoform |
|---|---|---|---|
| JVE015 | | | |
| JVE017 | yes | | |
| JVE044 | | | |
| JVE059 | | | |
| JVE103 | | | |
| JVE109 | | | |
| JVE114 | yes | yes | G3 |
| JVE127 | yes | | |
| JVE187 | yes | unknown | |
| JVE192 | yes | yes | G3 |
| JVE207 | | | |
| JVE222 | yes | yes | G3 |
| JVE241 | | | |
| JVE253 | yes | | |
| JVE367 | yes | | |
| JVE371 | yes | yes | unknown |
| JVE528 | yes | yes | G3 |
| JVE774 | yes | | |
| KP283T | yes | | |
| KP363T | | | |
| KP7038T | yes | | |

The amplicons obtained from the CRC cell lines JVE114, JVE192, JVE222 and JVE528 showed a strong homology with HLA-G3, when compared with the HLA-G3 mRNA sequence (ENST00000376815) (Figure 2C) and the coding sequence in order to evaluate mutations. In cell line JVE114 three single nucleotide polymorphisms (SNPs) were observed in the mRNA sequence. One SNP was located in exon 5 and the other

two were located in the 3'-UTR (indicated with red, figure 2C). Furthermore, in JVE114 a deletion in the first part of exon 7 was observed (represented by the empty box in figure 2C). In cell line JVE192 a SNP was observed in exon 5 and multiple known 3'-UTR variants were observed (indicated with red, figure 2C). Furthermore, an insertion of 14 base pairs was located in exon 7 (insertions are indicated by a green line in figure 2C). This insertion was originating from intron 6. Furthermore in JVE192 three single nucleotide insertions were observed in the 3'-UTR of exon 7 (indicated in green, figure 2C). However, these insertions were not observed in the coding sequence, since they were located in the 3'-UTR. In cell line JVE222 a "COSMIC" mutation (Catalogue Of Somatic Mutations In Cancer) was observed in exon 5 [29]. In the 3'-UTR one known and one unknown 3'-UTR variant was observed (indicated in red, figure 2C). Cell line JVE528 has three known SNPs. Furthermore, a deletion of exon 7 was observed in JVE528. JVE371 showed homology with HLA-G. However, in the results obtained by Sanger sequencing a substantial amount of non-matching and unknown base pairs were observed, which is most likely due to low amplicon concentrations. Therefore, we were not able to draw conclusion regarding the HLA-G isoform in JVE371. Also, most likely due to low amplicon concentration, the results obtained by Sanger sequencing of the visible band in cell line JVE187 showed very low signal peaks. Consequently, the obtained sequence was not reliable.



### PRESENCE OF HLA-G PROTEIN

To measure the presence of HLA-G protein, we performed flow cytometry as well as IHC. Cell surface expression of HLA-G1 was investigated by using mAb MEM-G/9 in flow cytometry. As expected, HLA-G1 was expressed on the JEG-3 cell line, used as a positive control. JEG3 cells were also reactive with W6/32 (pan beta-2 microglobulin-associated HLA class I). Although, the majority of the CRC cell lines were positive for W6/32 (Figure 3A) albeit in varying levels, they were all negative for HLA-G1 surface expression compared with the positive control JEG-3 (Figure 3B). These observations therefore reveal that the HLA-G1 isoform was not expressed in the CRC cell lines.

The CRC cell lines were embedded in paraffin (16 out of 21). We were able to collect associated paraffin-embedded tumor tissue of 18 patients. To evaluate HLA-G expression in the paraffin-embedded tissues by IHC, three different anti-HLA-G mAbs were used (4H84, MEM-G/1 and MEM-G/2. To avoid variation due to tissue heterogeneity, sequential sections were used in IHC using the different mAbs. An overview of the staining results and representative images of HLA-G staining with the three different mAbs are shown in Figure 4. As demonstrated in this figure, different staining patterns, regarding positive and negative stained tumor cells were observed between the three different HLA-G mAbs. For example, the paraffin-embedded cell line

KP283T was positive for 4H84 and MEM-G/1, but was negative for MEM-G/2. Cell line JVE253 very intensely stained with 4H84, but was negative for MEM-G/1 and MEM-G/2 (Figure 4B). No concordance was observed between positive stained tumor sections and those from the CRC cell lines derived from these tumors. In contrast, all anti-HLA-G mAbs showed a similar positive reactivity pattern in JEG-3 (Figure 4B) and in placenta tissue (data not shown).

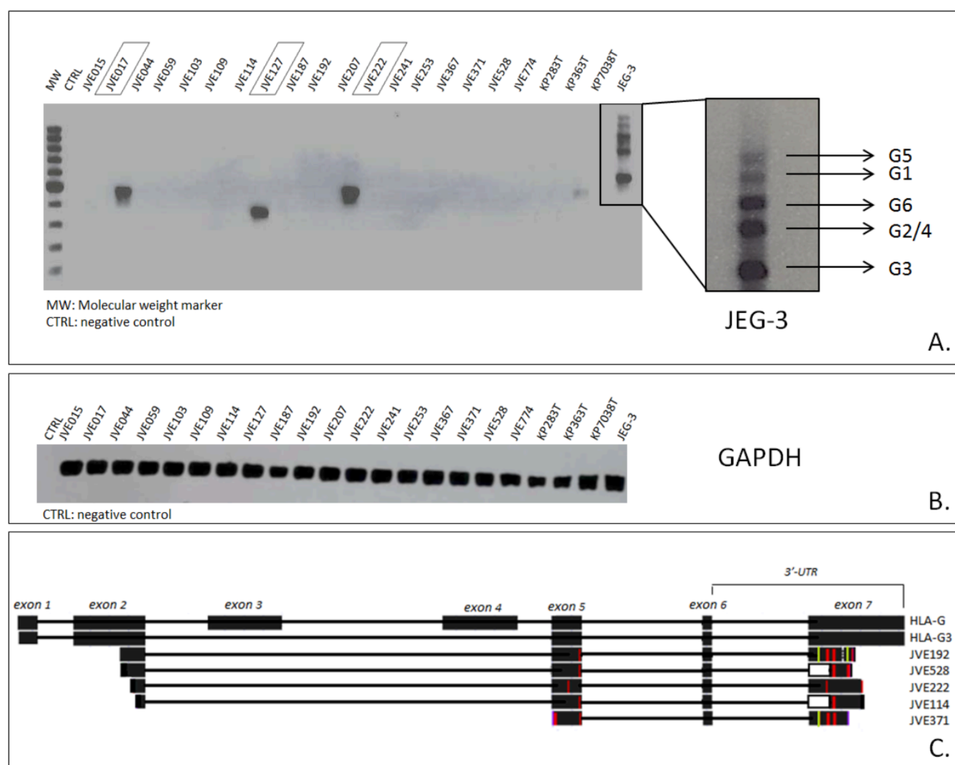


**Figure 2. HLA-G, mRNA expression. A.** The upper panel shows the results of a PCR performed with primer G.257 and with primer G.1225. As shown all different isoforms were detected in JEG-3. Furthermore, JVE017, JVE127 and JVE222 are showing positive amplicons. **B.** Panel B shows the results of a PCR performed with primers designed for the housekeeping gene GAPDH in the CRC cell lines. **C .** Alignment of the sequenced amplicons of the positive cell lines with HLA-G (ENST00000428701) and HLA-G3 (ENST00000376815) mRNA sequence. The amplicons of the cell lines JVE192, JVE528, JVE222, JVE 114 showed a strong homology to the HLA-G3 mRNA sequence. JVE371 displayed less homology to the HLA-G3 mRNA sequence. Red indicates a SNP at this position between the HLA-G3 sequence and the amplicons (two or more SNPs almost adjacent are appearing as a "bold" red line). Green indicates an insertion. Purple indicates that the obtained sequence extends beyond the end of the alignment. The empty box indicates a deletion.

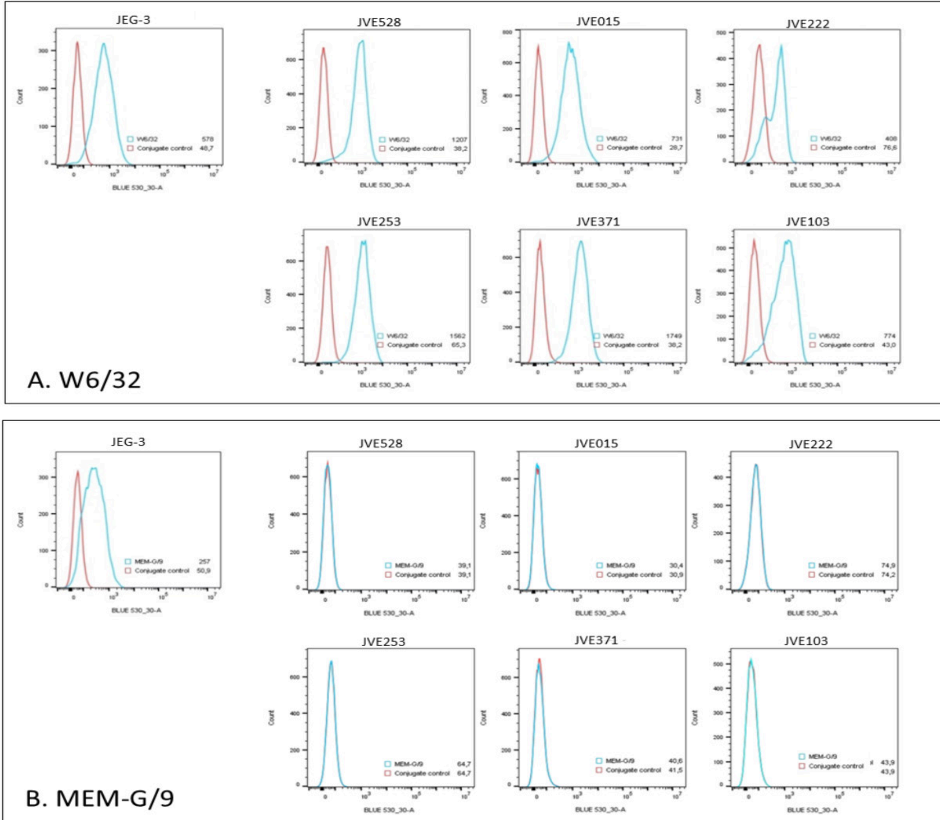




**Figure 3: HLA-G expression measured by flow cytometry.** Membrane expression of HLA-G measured by flow cytometry with mAb MEM-G/9. The mAb W6/32 recognizing HLA class I was used as a control. **A.** Representative histograms of flow cytometry performed on JEG-3 and CRC cell lines with mAb W6/32. JEG-3 and all CRC cell lines reacted positive with this antibody. **B.** Representative histograms of flow cytometry performed on JEG-3 and colorectal cancer cell lines with mAb MEM-G/9. Only JEG-3 was positive, while all CRC cell lines were negative

| Cell Line | 4H84 | MEM-G/1 | MEM-G/2 |
|---|---|---|---|
| JVE015 | N/A | N/A | N/A |
| JVE017 | - | - | - |
| JVE044 | + | + | - |
| JVE059 | N/A | N/A | N/A |
| JVE103 | - | - | - |
| JVE109 | N/A | N/A | N/A |
| JVE114 | - | - | - |
| JVE127 | - | - | - |
| JVE187 | - | - | - |
| JVE192 | - | - | - |
| JVE207 | - | - | - |
| JVE222 | + | - | - |
| JVE241 | - | - | - |
| JVE253 | + | - | - |
| JVE367 | - | - | - |
| JVE371 | - | - | - |
| JVE528 | + | - | - |
| JVE774 | N/A | N/A | N/A |
| KP283T | + | + | - |
| KP363T | - | - | - |
| KP7038T | - | - | - |
| JEG-3 | + | + | + |

| Tumor tissue | 4H84 | MEM-G/1 | MEM-G/2 |
|---|---|---|---|
| JVE015 | N/A | N/A | N/A |
| JVE017 | N/A | N/A | N/A |
| JVE044 | N/A | N/A | N/A |
| JVE059 | + | - | - |
| JVE103 | + | - | - |
| JVE109 | + | - | - |
| JVE114 | + | - | - |
| JVE127 | - | - | - |
| JVE187 | + | - | - |
| JVE192 | - | - | - |
| JVE207 | - | - | - |
| JVE222 | + | + | - |
| JVE241 | - | - | - |
| JVE253 | + | - | - |
| JVE367 | + | - | - |
| JVE371 | - | - | - |
| JVE528 | + | - | - |
| JVE774 | + | - | - |
| KP283T | + | + | + |
| KP363T | - | - | - |
| KP7038T | + | - | - |

N/A = Not Applicable ; (+) = Positive stained; (-) = Negative stained

A.



**Figure 4.** Reactivity patterns among three anti-HLA-G mAbs (4H84, MEM-G/1 and MEM-G/2) in sequential tissue sections of the cell lines and colorectal tumors**.** For the staining with MEM-G/1 and MEM-G/2 blocking with 5% goat serum was used. **A.** Overview of CRC cell lines showing positive or negative stained sections, for the three mAbs**,** (+) indicates positive staining, (–) indicates negative staining. **B.** Overview of CRC cell lines showing positive or negative stained

sections, for the three mAbs, (+) indicates positive staining (–) indicates negative staining. C. Representative images of different reactivity patterns. JEG-3 showed positive staining for all three mAbs. Cell line KP 2883T showed positive staining for 4H84 and MEM-G/1 and was negative for MEM-G/2. Cell line JVE 253 showed positive staining for 4H84 and was negative for MEM-G/1 and MEM-G/2. Sequential tissue sections of tumor tissue associated with JVE 253 showed positive staining for 4H84 and was negative for MEM-G/1 and MEM-G/2. Sequential tissue sections of tumor tissue associated with JVE 22 showed positive staining for 4H84 and MEM-G/1 and was negative for MEM-G/2.

# DISCUSSION

HLA-G has been of interest for the last decades as a possible immune tolerogenic molecule in several malignancies such as CRC. As a consequence HLA-G is considered as a potential target for immunotherapy strategies [30]. However, the results regarding HLA-G expression in cancer are controversial. Therefore, careful evaluation of HLA-G expression is necessary. In the current literature, several factors have been suggested to be essential for HLA-G expression such as the epigenetic status, miRNAs, tissue specific activators and polymorphisms [12]. It is generally accepted that long term culturing of cells can lead to changes in transcription and expression of specific genes. It was proposed that low passage CRC cell lines demonstrate a closer resemblance to the primary tumor source regarding HLA-G expression. Therefore a number of recently developed CRC cell lines, which were never studied before in the context of HLA-G, were analyzed for *HLA-G* DNA methylation of the minimal promoter and the CpG island-3, the presence of mRNA encoding HLA-G and expression of the HLA-G protein.

We analyzed the methylation status of the HLA-G gene at two separate locations. In comparison with HLA-G expressing JEG-3 cells, lower methylation levels were observed in the minimal promoter in 4 out of 21 of the CRC cell lines. Likewise, lower methylation percentages in CpG island-3 were also observed in 12 out of 21 CRC cell lines. More importantly, no correlation between mRNA expression and methylation levels of the *HLA-G* gene was observed. This observation is in line with the observation by *Holling et al.*, they analyzed the methylation pattern of the minimal promoter and a CpG rich area in exon 3 of the *HLA-G* gene in the HLA-G positive cell line JEG-3 and the HLA-G negative cell line JAR [21]. No significant difference in DNA methylation was observed in this study. Therefore, HLA-G methylation of these areas alone cannot predict HLA-G expression. These data suggest the involvement of other mechanisms in regulating transcription of the *HLA-G* gene, such as specific histone modifications

or miRNA [31, 32]. In this respect, especially miR-148/152 family members have proven to target the 3'-region of HLA-G resulting in downregulation of HLA-G expression [33]. Furthermore, members of the miR-148/152 family are regulated by DNA methylation of CpG islands and are thought to contribute to the regulation of expression of DNA methylation transferase 1 (DNMT1) [34]. Therefore, demethylating agents, used in cancer treatments, could influence the mechanism of action of these miRNAs.

After performing a RT-PCR multiple times followed by gel electrophoresis 13 out of 21 cell lines yielded a positive signal. We were able to sequence amplicons obtained from 6 cell lines. Four out of 6 of these sequenced amplicons displayed a strong homology with HLA-G3. The minor sequence variations in de coding region observed with HLA-G3 did not yield a stop codon and to our knowledge were corresponding with known single nucleotide polymorphisms. Furthermore, the observed deletions and insertions were located in the 3'-UTR. These observations therefore support the notion that these CRC cell lines potentially express functional HLA-G3. Furthermore, in one cell line a high degree of not matching nucleotides was observed suggesting that this cell line expresses a non-functional HLA-G3 protein. In a number of cases unknown mutations were found. In order to firmly establish the nature of these novel mutations a comparison with genomic DNA obtained from normal tissue of these CRC patients would be necessary. Unfortunately, normal tissue of these patients was not available.



Corresponding with the results obtained by RT-PCR, no HLA-G1 membrane expression was detected with flow cytometry in all CRC cell lines investigated. Some cell lines were positive for HLA-G protein expression visualized with IHC. For example JVE222, that proved to be transcribing HLA-G3, had low methylation levels compared to JEG-3 and showed to be positive in IHC for 4H84. The associated tumor tissue section was positive for 4H84 and MEM/G1. In contrast, cell line JVE044 which was in none of the tests positive for mRNA expression and highly methylated, showed positive IHC staining with 4H84 and MEM-G/1. Comparing HLA-G mRNA expression, methylation status and protein expression, the results did not correspond. Regarding IHC, no concordance was observed between positive stained cell lines and the corresponding tumor sections. This observation of no concordance between tumor tissue sections and tumor derived cells lines could be explained by the fact that tumors are heterogeneous in nature. Together with the notion that only a small number of cells within tumor sections were positive stained for HLA-G, makes it feasible that positive stained tumor sections could be negative for HLA-G in the associated cell line. More importantly, we observed discrepant expression profiles among various anti-HLA-G mAbs used in sequential tissue sections. This suggests that the different epitopes of HLA-G detected by 4H84, MEM-G/1 and MEM-G/2 are expressed differentially in colorectal tumor tissues as we

described previously [20]. In contrast, for all anti-HLA-G mAbs tested, positive staining patterns were observed in both JEG-3 and placenta tissue. This reveals the HLA-G specificity of these mAbs in placenta and the choriocarcinoma derived cell line JEG-3. However, in tumor cells and tissues conclusions based on immunohistochemistry alone have to be drawn very carefully. Especially literature based on one anti-HLA-G mAb should be interpreted with caution as the noted cross-reactivity and existence of non-specific binding may lead to an over-estimation of HLA-G expression in cancer [17, 19, 20]. Furthermore, it has been reported in HLA-G cancer research, that only low levels of HLA-G expression are present in tumors [35]. Low expression of HLA-G could be an explanation of our findings regarding the mRNA expression of HLA-G. Except for HLA-G3, the levels could have been below our detection level. This notion indicated that some HLA-G isoforms might be more prominent in some specific tumor types. For example, in pregnancy disorders it is suggested that smaller HLA-G isoforms are expressed as a substitute in situations in which HLA-G1 is altered [36]. Perhaps, such substitution mechanism could be extrapolated to tumor situations. In tumor cells that do not express HLA class I, a well-known mechanism to escape anti-tumor immunity, expression of smaller HLA-G isoforms might still occur [18, 37]. In this study HLA-G3 mRNA was found in CRC cell lines. This HLA-G isoform only contains the α1 domain. The α1 domain, common to all HLA-G isoforms, is suggested to interact with the KIR2DL4 receptor and thereby inhibiting NK cell function [38, 39]. However, contradictory evidence for the HLA-G/KIR2DL4 interaction has been published as well [40, 41]. Therefore, the functional consequences of the HLA-G3 expression in CRC remains to be elucidated.
In conclusion, HLA-G has been proposed as an interesting and promising protein in cancer research. However, based on the results of our study it is evident that methods utilized in this field are not selective enough to detect all aspects of HLA-G expression in CRC.

In the results of this study no strong association between *HLA-G* DNA methylation patterns and HLA-G expression was observed. Furthermore, we argue that HLA-G might be expressed differently in specific tumors types or some HLA-G isoforms might be more prominent in some specific tumor types. For CRC it could be HLA-G3. Many of the present studies mainly aim at investigating G1 and G5 isoforms. However, the discovery of a null allele (G*01:05N), resulting in aberrant synthesis of the G1, G5 and G2 isoforms, urges for more research regarding the importance of other isoforms [42]. Therefore, to make further steps ahead, a shifted study focus towards all isoforms of HLA-G in conjunction with increasing sensitivity of the methods available is a prerequisite.

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# Chapter 7

## HLA-G protein expression in colorectal cancer evaluated by immunohistochemistry and western blot analysis: its expression characteristics remain enigmatic

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# ABSTRACT

HLA-G protein expression could play a role in evasion of tumor immune surveillance. Accumulating evidence demonstrates that HLA-G is expressed in different types of malignancies, including colorectal cancer (CRC). The purpose of the current study was to further unravel whether HLA-G protein expression could play a role in immune evasion of CRC. Therefore, to firmly establish HLA-G protein expression, eight early passage human CRC cell lines and five human rectal cancer tissues were analyzed by western blot analysis. The results obtained by western blot analysis were compared with immunohistochemistry on tumor tissue sections of the same patient. Furthermore, multiple monoclonal antibodies (mAbs), 4H84, MEM-G/1 and 5A6G7, targeting HLA-G were used to unravel staining patterns. We showed that results obtained with immunohistochemistry did not correlate with protein expression detected by western blot analysis, using three different HLA-G targeting mAbs. Furthermore, with respect to the specificity of the mAbs employed, additional immune reactivity was detected using the mAbs MEM-G/1 and 5A6G7 in western blot analysis with K562 control cell lines overexpressing HLA-A2 or HLA-G, all tumor tissues and in two out of eight CRC cell lines. Based on the current study and our previously reported results, we conclude that claiming HLA-G plays a role in immune modulation of CRC seems premature, as results from anti-body based detection of HLA-G protein remain inconclusive. Until the time that detection of HLA-G is sensitive enough to detect all aspects of HLA-G expression in biological samples, rather than transfected cells or long time cultured cell lines, conclusions should be drawn with great care.

# INTRODUCTION

In 2011, evasion of immune recognition was added as an additional hallmark to the well-known six hallmarks of cancer [1,2]. Currently, escaping the host's defense immunity, by the concept of cancer immune-editing, is widely accepted. To completely unravel the complex mechanisms that contribute to immune defense evasion of the host, is a major challenge. During the past decade the non-classical HLA class I molecule HLA-G, has been of interest as a possible immune tolerogenic molecule in cancer. As a result of its proposed immunosuppressive capabilities, HLA-G protein expression could play a role in tumor immune surveillance. Notably, altered HLA class I expression is a well-known mechanism in escaping anti-tumor immunity [3-6].

In non-pathological conditions the HLA-G protein was found to be expressed in immune privileged sites, for example extravillous trophoblast cells of the placenta. Its expression in these cells of the placenta plays an important role in establishing maternal-fetal immune tolerance [7-9]. The expression of HLA-G protein by fetal trophoblast cells has been proved by using different biochemical techniques together with many different anti-HLA-G monoclonal antibodies (mAbs) [10,11]. Though, despite the impressive expansion of knowledge regarding HLA-G, many questions remain unanswered [12].

HLA-G protein expression has been observed in a wide range of human tissues and in several malignancies, including colorectal cancer (CRC) [13-15]. This tumor driven, *de novo* expression of the HLA-G protein, which possibly could contribute to evasion of the host's immune surveillance, has been associated with a poor clinical outcome in patients with CRC, warranting further research into its expression patterns in cancer [13,16]. HLA-G is considered an immune checkpoint molecule, and as a consequence, *de novo* HLA-G tumor expression has been ascribed potential as a immunotherapy target [17,18]. However, among and within different tumor types discrepancies regarding HLA-G expression were reported. In CRC, HLA-G protein expression was found in 20% of the patients by *Zeestraten et al.* [19], whereas Guo *et al.*[20] reported HLA-G protein expression in 72% of their patient cohort. Notably, even higher percentages were described by *Kirana et al* [21], who observed HLA-G protein expression in 86% of their CRC cohort. Detecting HLA-G expression with the use of immunohistochemistry (IHC) is a widely used technique, but remains controversial [22-24]. Due to known cross-reactivity of mAb 4H84, it has been suggested to use multiple mAbs to evaluate HLA-G expression by IHC [22,23]. However, most studies based on IHC used a single mAb, usually 4H84, and therefore should be interpreted with caution. Moreover, different discrepant expression profiles in sequential CRC tissue sections have been observed [24,25]. Therefore, it seems

premature to conclude HLA-G is expressed in a pathological condition such as CRC. To firmly evaluate HLA-G expression in CRC additional molecular and biochemical analyses are required.

In a previous study, we evaluated HLA-G expression in 21 low passage CRC cell lines and in the corresponding primary tumor tissues [25]. Flow-cytometry, RT-PCR and IHC with 3 different mAbs was performed. Of the known HLA-G isoforms, only mRNA showing strong homology with HLA-G3 was detected, albeit at very low levels and in only 5 out of 21 CRC cell lines. In accordance with the RT-PCR results, HLA-G1 was not detected by flow-cytometry performed on the same CRC cell lines. Moreover, IHC of the CRC tissue matching the cell lines showed different staining patterns with the different anti-HLA-G mAbs.

The purpose of the current study was to further unravel whether HLA-G protein expression plays a role in immune evasion of CRC. Therefore, early passage CRC cell lines and CRC tissues were analyzed using western blot for HLA-G protein expression. In addition, the results obtained by western blot analysis were compared with IHC on corresponding frozen tumor tissue sections. Furthermore, multiple mAbs targeting HLA-G were used to unravel binding patterns. We showed that results obtained with IHC did not correlate with protein expression detected by western-blot analysis, using three different HLA-G targeting mAbs.



# MATERIALS AND METHODS

## CELL AND CULTURE CONDITIONS

The myelogenous leukemia cell line (K562), was transfected with a single HLA heavy chain i.e. HLA-G1 or HLA-A2, in pLNCX (Ampicillin and Neomycin resistant), and named K562-G1 and K562-A2 respectively. K562 wild type (K562-WT), transfected with empty pLNCX vector, was used as a negative control. The transfected cell lines were previously described and were a kind gift of Y.M. Zoet from the Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands [26]. All K562 cell lines were cultured in RPMI-1640 (Gibco™,Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal calf serum (FCS) and 2-mM L-Glutamine. The transfected K562 cells were cultured with G418 (neomycin derivative, final concentration: 200 µg/ml; Invitrogen, Groningen, The Netherlands). The HLA-G expressing choriocarcinoma cell line JEG-3, was cultured in DMEM (Gibco™) with 10% FCS.

Early passage CRC cell lines were established from primary CRC tumors and colorectal liver metastasis at the department of Pathology, Leiden University Medical Center, Leiden, The Netherlands. The cell lines have been characterized for cancer-related mutations by Boot et al. [27]. These CRC cell lines were previously investigated for the presence of mRNA encoding the HLA-G protein [25]. Four cell lines (JVE114, JVE192, JVE222 and JVE528) were previously identified to express mRNA encoding the HLA-G3 protein, albeit at very low level. Furthermore, four CRC cell lines negative for mRNA encoding HLA-G protein were selected as well (JVE044, JVE103, JVE207, JVE241). The selected early passage CRC cell lines were cultured in Dulbecco's Modified Eagle medium (DMEM)/F12 (Gibco™) with 10% FCS. Culturing conditions were the same for all the cell lines ($37^0$C, 5% $CO_2$).

### ANTIBODIES

HLA-G targeting mouse mAb 4H84 (Exbio, Czech Republic) and MEM-G/1 (Abcam, Cambridge, UK, ab 7759), both recognizing all HLA-G isoforms (according to the manufacturer), were used to assess HLA-G expression on frozen tumor tissue sections. Furthermore, the mAb clone 5A6G7 (Sigma-Aldrich, Saint Louis, USA) detecting soluble HLA-G (sHLA-G) was used. To visualize HLA-class I mAb HCA2 was used (kindly provided by Prof. Dr. J. Neefjes, Leiden University Medical Center, Leiden**,** the Netherlands). The HCA2 mAb recognizes all HLA-A chains (except HLA-A24), some HLA-B, HLA-C, HLA-E, HLA-F, and HLA-G chains [28,29]. For protein loading control in western blot analysis the mAb Anti-alpha-Tubulin (Abcam, ab7291) was used.

### CELL AND TISSUE LYSIS, PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

For preparation of protein extracts, 30 sections of 10μm frozen rectal cancer tissues were crushed with a mortar under ice cold conditions and lysed with NP-40 lysis buffer (50 mM Tris-base pH=7.8, 150 mM NaCl, 5mM ethylenediaminetetraacetic acid, 0.5% NP-40, 1x HALT™ Protease & phosphatase inhibitor Cocktail, EDTA-free, Thermo scientific). Cell lines were washed three times with ice cold PBS. Next, cell pellets were collected and lysed with NP-40 lysis buffer, 100 μl per 1 × $10^6$ cells/ml, for 30 min. After centrifugation at 12.000 rpm at 4°C for 20 min, supernatants were collected and protein concentration was determined using the Pierce™ BCA protein assay (ThermoFisher Scientific, Waltham, MA USA). All samples were heated for 5 min at 95°C in Laemmli Sample Buffer (Bio-Rad Laboratories, Hercules, California, USA ) before loading. Cell lysate aliquots were separated in 12% SDS-PAGE (mini-PROTEAN® TGX™ precast gel, Bio-Rad). Proteins were electro-blotted onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad) and blocked by incubation with TBST ((20mM Tris ( pH=7.6), 150mM NaCl and 0.2% Tween-20)) containing 5% non-fat dry milk for 1hr at room temperature. After blocking, membranes were washed in TBST three times for 5

min and then probed with the mAbs overnight at 4°C. Subsequently, the membranes were washed with TBST and incubated for 1hr at room temperature with rabbit-anti mouse-HRP (Dako, Glostrup, Denmark) in TBST containing 5% non-fat dry milk. Finally, membranes were washed three times and developed with Clarity™ Western ECL substrate (Bio-Rad), followed by imaging with ChemiDoc™ MP (Bio-Rad).

### IMMUNOHISTOCHEMISTRY

To assess HLA-G expression on frozen tissue sections, HLA-G targeting mouse mAbs 4H84 and MEM-G/1 were used. Furthermore, mAb 5A6G7 recognizing sHLA-G and HCA-2 was used to detect HLA class I. Tissue sections of 4 μm were cut from the fresh frozen tumor tissues. A standard IHC protocol using the Envision$^{+}$ (Dako) was performed. Briefly, slides were fixed in acetone for 10 min and washed twice with Phosphate Buffered Saline (PBS). Followed by blocking with BLOXALL (Vector laboratories, California, USA) for 10 min. Thereafter, tissue sections were washed two times for 5 min with PBS. Nonspecific protein binding was blocked with 5% normal goat serum (Dako) for 20 min. The sections were incubated, at pre-determined optimal dilutions, for 1hr at room temperature with the primary antibody (4H84, MEM-G/1, 5A6G7 or HCA-2). Incubation was followed by washing the tissue sections, three times for 5 min in PBS followed by 30 min incubation with EnVision+ System-HRP anti-mouse (Dako). The staining was completed by 10 min incubation with 3,3'-diaminobenzidine (DAB)+ Substrate-Chromogen System (Dako). Next, sections were washed twice in demineralized water and counterstained with Haematoxylin. Finally, sections were dehydrated and mounted in Pertex**.** For anti-HLA-G mAbs, placenta tissue was used as a positive control. Sections serving as negative controls underwent the entire protocol except primary antibody incubations. Expression of HLA-G was evaluated as positive or negative.



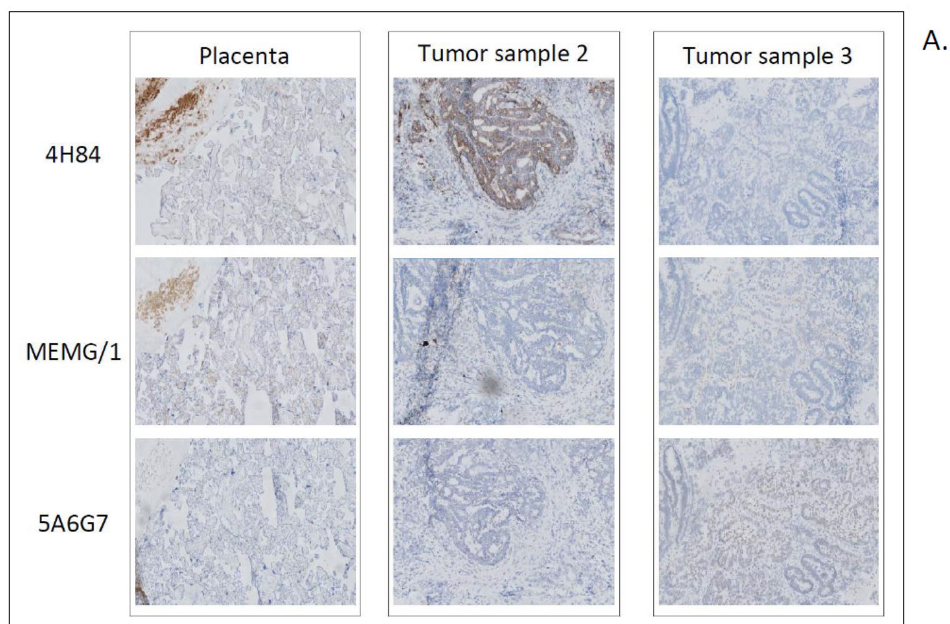
## RESULTS

### IMMUNOHISTOCHEMISTRY OF TUMOR TISSUE SECTIONS

To evaluate HLA-G expression in fresh-frozen tissue sections by IHC, two mAbs detecting all HLA-G isoforms were used, namely 4H84 and MEM-G/1. Furthermore, the mAb 5A6G7 was used, detecting soluble HLA-G5/HLA-G6 by recognizing the retrained intron 4 (according to the manufacturer). Since sequential tissue sections were used, variation due to tissue heterogeneity was not expected. Representative images of the staining patterns are shown in Figure 1. In line with previous reported results [24,25], both 4H84 and MEM-G/1 (detecting all HLA-G isoforms) showed a similar positive reactivity

pattern in placenta tissue (Figure 1). In contrast, tumor tissue sections stained with both 4H84 and MEM-G/1 showed varying staining patterns dependent on the mAb used. For example, tumor sample 2 was positive for 4H84, but no reactivity with MEM-G/1 was observed. Tumor sample 3 did not reveal any reactivity with 4H84 or MEMG/1.

With respect to sHLA-G, no reactivity was observed with 5A6G7 in placental tissue. Reactivity for 5A6G7 was however observed in tumor sample 3. The results of all IHC analyses are summarized in Figure 1, panel B.




B.

| | Immunohistochemistry | | |
|---|---|---|---|
| | 4H84 | MEM-G/1 | 5A6G7 |
| Tumor 1 | - | - | - |
| Tumor 2 | + | - | - |
| Tumor 3 | - | - | + |
| Tumor 4 | - | - | - |
| Tumor 5 | + | - | + |

**Figure 1. Representative images of immunohistochemistry with 4H84, MEM-G/1 and 5A6G7**. **A.** In the left panel sequential placenta tissue sections positively stained with 4H84 and MEM-G/1 which showed the same reactivity pattern. Placenta tissue sections stained negative for 5A6G7. Sequential tissue sections of tumor sample 2 was positive for 4H84, negative for MEM-G/1 (both detecting all HLA-G isoforms) and negative for 5A6G7. The right panel, showing sequential tumor tissue section of tumor sample 3, was negative for 4H84 and MEM-G/1 and showed positive staining with 5A6G7. **B.** Summary of the immunohistochemistry analysis.

**WESTERN BLOT ANALYSIS OF CRC CELL LINES**

Low levels of HLA-G expression in tumors have been reported [25,30]. Therefore, a dilution experiment was performed to establish the lowest HLA-G expression level that we were able to detect by western blot analysis with 4H84 and MEM-G/1. The HLA-G overexpression cell line K562-G1 was serially diluted with the HLA negative cell line K562-WT. As shown in Figure 2, we were able to detect HLA-G expression as low as 5% of HLA-G expressing cells in a mixture for 4H84 and 10% for MEM-G/1. Therefore, K562-G1-10% was used as a positive control, in addition to K562-G1-100%. Next, we investigated HLA-G expression in the selected early passage CRC cell lines which were found to express very low levels of mRNA encoding HLA-G3 (JVE114, JVE192, JVE222 and JVE528) and four CRC cell lines, lacking expression of any HLA-G isoform (JVE044, JVE103, JVE207, JVE241) [25].

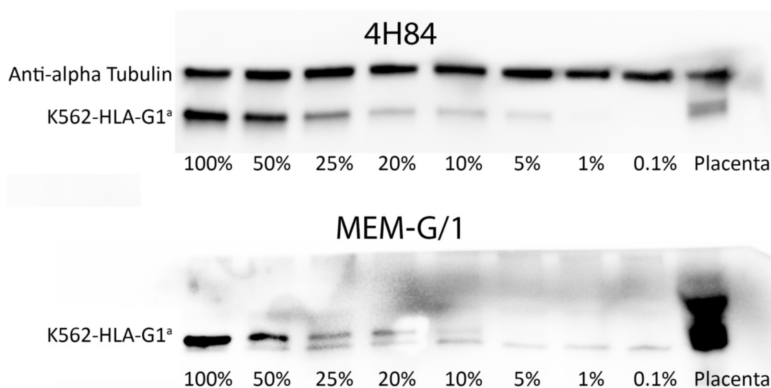



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**Figure 2. Dilution experiment.** Representative image of the dilution experiment, to establish the lowest HLA-G expression level which we were able to detect by western blot analysis with 4H84 and MEM-G/1. The HLA-G overexpression cell line K562-G1 was diluted with the HLA-G negative cell line K562-WT. As shown in this figure we were able to detect HLA-G expression as low as 5% for 4H84 and 10% for MEM-G/1. For MEM-G/1 a smaller, non-specific, band was detected as well, visible in every dilution.

As shown in Figure 3A, all CRC cell lines clearly showed expression of HLA-A2 molecules, when western blot analysis was performed with mAb HCA-2. HCA-2 is also known for reacting with HLA-G. As shown in Figure 3A a clear positive band at approximately 35 kDa, corresponding with the molecular weight of HLA-G, was observed for the positive control cell line K562-G1. Furthermore, a clear positive band, corresponding with the size of HLA-A2, was observed in control cell line K562-A2 at approximately 39 kDa. No HLA-G expression was detected in any of the CRC cell lines using mAb HCA-2.

For the detection of HLA-G with mAbs 4H84 and MEM-G/1, K562-G1 served as a positive control and HLA-G1 protein expression was clearly present (Figure 3A). The JEG-3 choriocarcinoma cell line (originating from trophoblast cells), known for expression of soluble HLA-G, was used as a positive control for western blot analysis using mAb 5A6G7. The staining pattern revealed a clear positive band at approximately 33kDa corresponding in size with HLA-G6 (Figure 3B). As shown in Figure 3A and B, western blot analysis using 4H84, MEM-G/1 or 5A6G7, did not detect any HLA-G isoforms in CRC cell lines corresponding in size with those expressed in the control cell lines K562-G1 or JEG-3.

Notably, western blot analysis using MEM-G/1, all K562 control cell lines and also in some CRC cell lines showed additional immune reactivity at a molecular weight of approximately 32kDa, (indicated by the (*) in Figure 3A). Additional immune reactivity ranging from 36-175kDa was detected with 5A6G7 as well (Figure 3B).

**WESTERN BLOT ANALYSIS WITH RECTAL CANCER TISSUE**

Figure 4 shows the western blot analysis for rectal cancer protein lysates, derived from 5 frozen primary tumor tissues. As shown in Figure 4A, tumor tissue sections of all these 5 tumors stained positive for HCA-2. When using the mAb 4H84 no clear detectable HLA-G encoding proteins could be detected in these rectal tumor tissue samples. However, some reactivity with an estimated molecular weight of 37kDa in tumor 2, 32kDa in tumor 4 and 5 and 41kDa in all tumors was visible indicated by (*) (when exposure time was increased, bands became more visible; data not shown). The size of these additional proteins did not correspond with any of the known HLA-G isoforms. Importantly, the observed immune reactions in the western blot analysis did not correspond with the IHC staining patterns in matching tumor tissues (Figures 1 and 4).
Similarly, in the western blot analysis using MEM-G1, none of the rectal tumor tissue samples revealed expression of proteins corresponding in size with HLA-G.

Like for the 4H84 mAb, the western blot analysis using MEM-G/1 also revealed additional immune reactivity with a molecular weight of approximately 32kDa and 48kDa in all controle cell lines and in all CRC tissues.

For western blot analysis using 5A6G7 all tumor tissue samples did not show immune reactivity corresponding in size to those detected in JEG-3 (Figure 4B). More importantly, intense additional immune reactivity was observed with proteins with a molecular weight varying from 36-175kDa in the K562 control cell lines. In all tumor tissues addition immune reactivity at approximately 44kDa were detected.

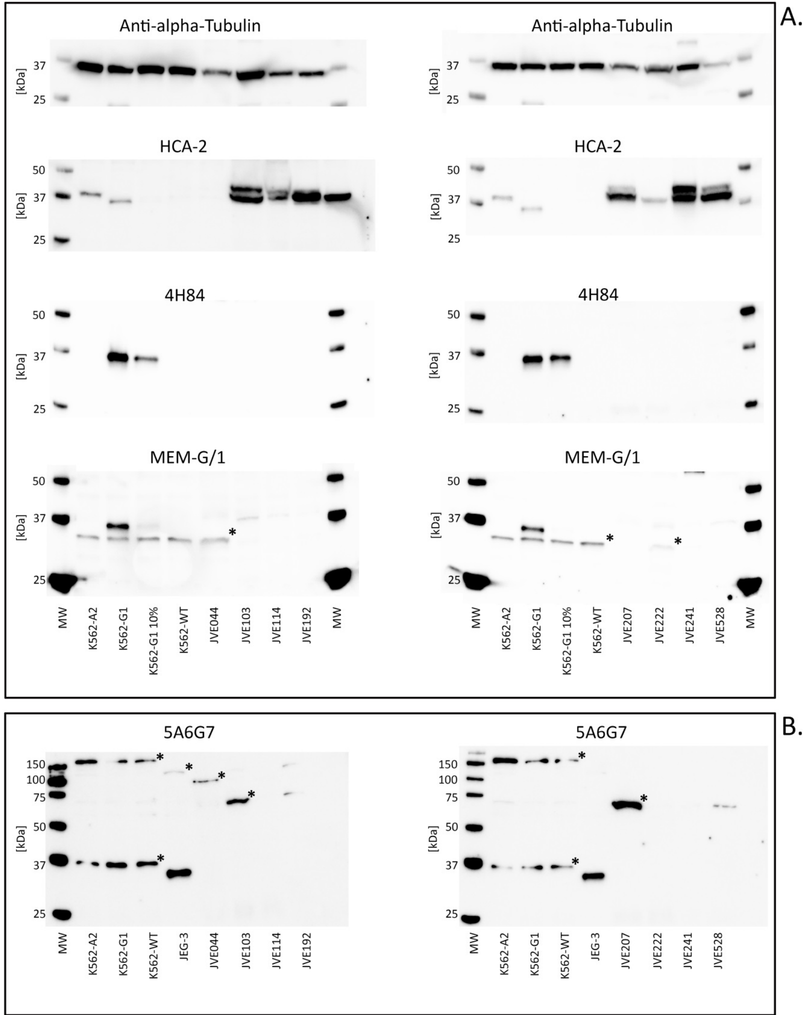


**Figure 3. Western blot analysis for colorectal cancer cell lines protein lysate.**

As shown in panel **A**, all cell lines were similar in expressing Anti-alpha-Tubulin, used as a loading control. All CRC cell lines were expressing HLA-A2. Furthermore, HLA-G was clearly detected by HCA2 in K562-G1, illustrated by the difference in molecular weight of the detected protein. No bands corresponding in size with HLA-G1 were detected with HCA-2 in the CRC cell lines. For the detection of HLA-G with 4H84 and MEM-G/1, cell line K562-G1 served as a positive control and a clear expression of the HLA-G protein at approximately 35kDa was shown. In all CRC cell lines, HLA-G expression was absent using mAb 4H84. For mAb MEM-G/1, K562-G1 showed a clear positive band. In all CRC cell lines HLA-G expression was absent, using mAb MEM-G/1. In all K562 control cell lines and in JVE044 and JVE222 additional immune reactivity with a molecular weight of approximately 32kDa, was detected indicated by (*).

In the panel **B** the results obtained with mAb 5A6G7 are shown. JEG-3 was clearly positive, whereas

all CRC cell lines sHLA-G expression was absent. Additional immune reactivity was detected for 5A6G7 for proteins with a varying molecular weight, indicated by (*).

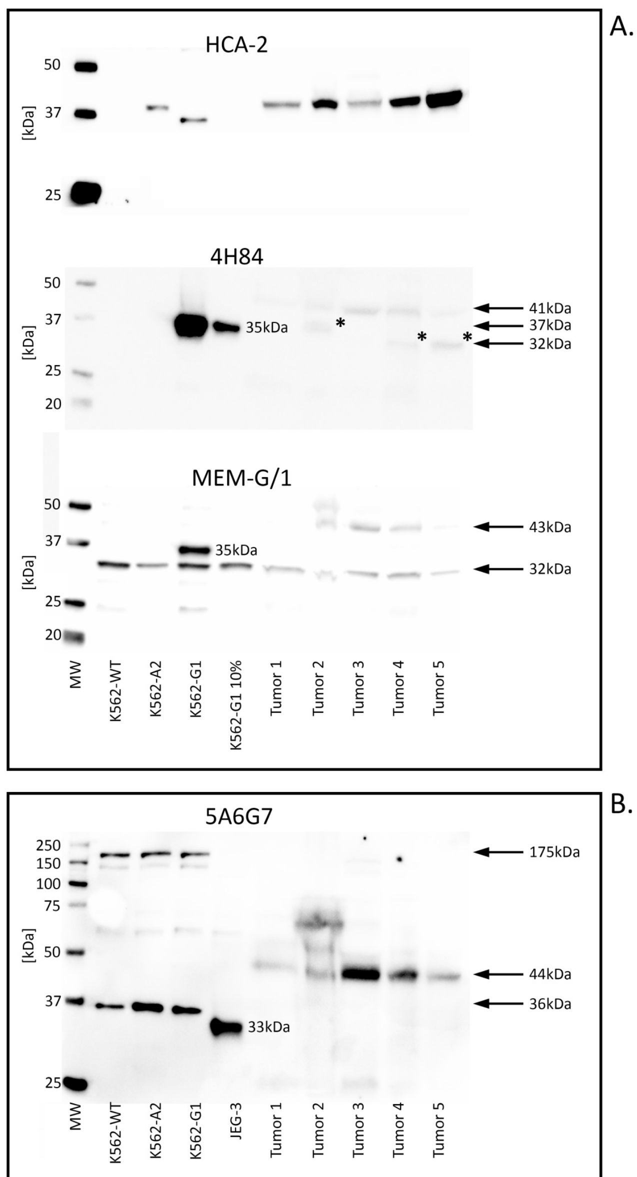


**Figure 4. Western blot analysis for rectal cancer tissue protein lysate.**

The figure demonstrates western blot analysis for rectal cancer protein lysates, derived from frozen primary tumor tissue. As shown in panel **A.** all tumor tissue sections were positive for mAb HCA-2. For mAb 4H84 positive bands were detected in K562-G1 and K562-G1 10%. No clear visible bands were detectable in the tissue samples compared with positive control K562-G1 and K562-G1 10%.

However, some reactivity was visible in tumor sample 2 (37kDa), tumor sample 4 (32kDa), and tumor sample 5 (32kDa), indicated by (*), (when the exposure time was adjusted bands became clearer, data not shown). Furthermore, for all tumor samples immune reactivity was observed at an estimated molecular weight of 41kDa. Using MEM-G/1, a clear positive band was detected in K562-G1 at approximately 35kDa. In none of the tumor tissue samples the HLA-G protein expression could be detected with MEM-G/1. Notably, using MEM-G/1, in all control cell lines and in all rectal cancer additional immune reactivity with a molecular weight of approximately 32kDa was detected. In the tumor samples a protein of approximately 43kDa was detected as well.
Using mAb 5A6G7, panel **B,** JEG-3 was clearly positive at approximately 33kDa, while all rectal tumor tissue samples did not show positive staining of proteins corresponding in size with JEG-3. More importantly, additional immune reactivity was detected for 5A6G7 with proteins with a varying molecular weight.

Similar results were seen in the tumor samples. For example IHC of tumor sample 2 showed intensive staining using mAb 4H84 while negative for MEM-G/1 and 5A6G7, but western blot, showed no clear bands corresponding with HLA-G. Likewise, tumor sample 3 stained positive with IHC using mAb 5A6G7, but no bands were detected corresponding in size with sHLA-G detected in JEG-3.

## DISCUSSION



In the present study, HLA-G protein expression in CRC was studied in more detail than in any of the previous studies. The results of our studies show that in early-passage CRC cell lines and primary rectal tumor tissues, no clear HLA-G protein expression could be detected, using mAbs 4H84, MEM-G/1 or 5A6G7 in western blot analysis. The size of the immune reactive proteins in the rectal tumors did not correspond with the size of the HLA-G protein observed in K562-G1 or in JEG-3. In contrast with the results obtained by western blot analysis, strong positive IHC staining patterns in matching tumor tissue were observed.

However, no concordance was observed between the results obtained by western blot analysis and IHC. Additionally, in sequential fresh frozen tissue sections stained with various HLA-G detecting mAbs, discrepant expression profiles were observed. This suggests non-specific binding. However, similar analyses with placenta tissue using 4H84 and MEMG/1 showed that the results of the IHC and the western blot analysis were consistent. This shows the specificity of these mAbs for HLA-G expression in placenta

tissue, but not for rectal cancer tissue. Furthermore, in all K562 control cell lines, in all tumor tissues and in two out of eight CRC cell lines additional immunoreactivity with a varying molecular weight was detected using MEM-G1 and 5A6G7 in western blot analysis. Consequently, it could be debated whether these mAbs are in fact detecting HLA-G in some tumor tissue samples. In summary, for CRC cell lines and rectal tumor tissues results obtained by western blot analysis did not correspond with the results obtained by IHC. This reveals the existence of non-specific binding, which could result in false positive recognition of HLA-G expression in CRC. As a consequence this may lead to an over-estimation of HLA-G expression in cancer, especially in studies using solely IHC.

The possibility that *de novo* expression of the HLA-G protein by tumor cells is present to escape immunosurveillance is fascinating, but considerable controversy exists. In a study by *Real et al.* [31] analyzing 50 solid tumors and 31 tumor cell lines of different origin, no HLA-G protein expression was detected, despite the presence of mRNA encoding HLA-G, although at several orders of magnitude lower than mRNA encoding classical HLA class I. They proposed that HLA-G protein expression could be under very strong post-translational control. The study by *Real et al*. corresponds with our previous findings of extremely low levels of mRNA encoding the HLA-G3 protein in some of the JVE cell lines [25], while no corresponding HLA-G protein expression could be detected by western blot analysis in this study. These findings suggests that the role of HLA-G protein expression in escaping immunosurveillance by NK cells in colorectal cancer, if any, will only be minor. We therefore feel that caution should be taken in literature describing solely IHC experiments performed with one mAb only, as the noted cross-reactivity with proteins other than HLA-G, may lead to false interpretation of HLA-G protein expression [23,32]. Accordingly, it could be proposed that conclusions based on IHC alone should be drawn very carefully.



Based on the current study in combination with our previous reported results [25], we conclude that the role of HLA-G as immune modulator in CRC is premature. HLA-G is considered as an immune checkpoint molecule and studied as a potential target for immunotherapy. Until the time that detection of HLA-G is selective enough to detect all aspects of HLA-G expression in biological samples, rather than transfected cells or long time cultured cell lines, therapeutic applications involving HLA-G will remain enigmatic.

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# PART III

## Genetic and epigenetic biomarkers in colorectal cancer

# Chapter 8

## Tumor LINE-1 methylation level in association with survival of patients with stage II colon cancer

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*International Journal of Molecular Sciences. 2016 Dec 27;18(1)*

## ABSTRACT

Genome-wide DNA hypomethylation is associated with a worse prognosis in early-stage colorectal cancer. To measure genome-wide DNA methylation levels, long interspersed nucleotide element (LINE-1) repeats are used as surrogate marker. Cohort studies on the clinical impact of genome-wide DNA methylation level in patients with early-stage colon cancer only, are currently lacking. This study aimed to investigate the prognostic value of LINE-1 methylation in a stage II colon cancer cohort (n=164). Manual needle microdissection of tumor areas was performed on formalin-fixed paraffin-embedded tumor tissue sections followed by DNA extraction. Bisulfite converted DNA was used to assess tumor LINE-1 methylation level by qPCR. Patients with LINE-1 hypomethylated tumors had a significantly worse overall survival compared to patients with a higher level of LINE-1 tumor DNA methylation (HR 1.68, 95%CI 1.03-2.75; *P*=0.04). This effect was more prominent in patients aged over 65 years (HR 2.00, 95%CI 1.13-3.52; *P*=0.02), although the test for age interaction was not significant. No significant effect on recurrence-free survival was observed. Based on these results, tumor LINE-1 hypomethylation is associated with a worse overall survival in stage II colon cancer. Whether the origin of this causation is cancer-specific or age-related can be debated.

# INTRODUCTION

Colorectal cancer (CRC) is one of the most commonly diagnosed malignancies across the world [1]. During the past three decades, CRC has been extensively studied regarding prognostic and predictive biomarkers, in order to establish more personalized treatment strategies. Currently, the first CRC biomarkers are entering clinical use, such as *RAS, BRAF*, and microsatellite mutational status. Clinically relevant biomarkers can be found at different molecular levels, and the role of epigenetics in carcinogenesis has become a focus in cancer research during the past decade. Genome-wide DNA hypomethylation is an important epigenetic alteration in cancer, including CRC, and is assumed to be an early event in the carcinogenesis and contributes to genomic instability [2,3]. The methylation status of long interspersed nucleotide element (LINE-1) repeats is used as surrogate marker to indirectly measure global DNA methylation [4]. LINE-1 repeats are present on most of the chromosomes and make up approximately 17% of the human genome [5]. Furthermore, LINE-1 has retrotransposition activity, and upon hypomethylation LINE-1 can be reversed transcribed into DNA sequences and transpose throughout the genome. Thereby, LINE-1 can contribute to gene disruptions and genomic instability, one of the hallmarks of cancer [6,7]. Currently, tumor LINE-1 hypomethylation is intensively studied and has been observed in almost all human cancer types [8,9]. Regarding CRC, LINE-1 hypomethylation is thought to be associated with a worse prognosis, supporting its role as prognostic biomarker [10]. In addition, studies have indicated that tumor LINE-1 methylation levels correlate with tumor stage; a decrease in LINE-1 methylation, resulting in hypomethylation, was associated with more advanced disease stages [11,12]. A correlation between survival in CRC patients and tumor LINE-1 methylation status in more advanced disease stages has not been observed [12-14]. In the current literature, tumor LINE-1 hypomethylation in relation to clinical outcome in CRC was predominantly studied in cohorts consisting of both colon and rectal cancer. Research has provided evidence that rectal cancers differ significantly from colon tumors [15,16]. For example; rectal cancer has less microsatellite instable (MSI) tumors and fewer *BRAF* mutations when compared with colon cancer [17,18]. Many studies have supported the "two types of CRC's" hypothesis resulting in a more definitive separation of colon and rectal cancer for scientific research and treatment strategies. For early-stage rectal cancer, the study of Benard *et al.* showed that LINE-1 hypomethylation was associated with a worse overall survival (OS) and higher tumor recurrence rates [14]. Large cohort studies focusing on patients with early-stage colon cancer are currently lacking. This study aimed to investigate the role of tumor LINE-1 methylation level and its relation to clinical outcome in stage II colon cancer specifically. Selecting high-risk patients with colon cancer by prognostic biomarkers is of great importance in order to avoid over-, or under treatment. Since

an age-related global hypomethylation has been observed in the colon [19,20], analyses stratified by age were performed in this study. Furthermore, it is uncertain whether the proposed prognostic value of LINE-1 hypomethylation is altered by MSI [21,22]. For this reason, the *MLH1* methylation status was determined, as *MLH1* promoter methylation is the most frequently observed cause of MSI in sporadic colon tumors [23].

# MATERIALS AND METHODS

## PATIENT SELECTION

Formalin-fixed paraffin-embedded (FFPE) tumor tissues were collected from patients with stage II colon cancer who underwent radical resection of the primary tumor between 1991 and 2011 at the Leiden University Medical Center. Patients with a history of cancer other than basal cell carcinoma, patients that received radiotherapy and/or chemotherapy prior to resection, patients with multiple synchronous colon tumors and patients with rectal cancer were excluded. Based on availability of paraffin tissue blocks, 181 patients were included. Clinical, pathological and follow-up data were collected in a retrospective manner from hospital records. Patient information was anonymized and de-identified prior to analysis. This research was performed according to the national ethical guidelines ( "Code for Proper Secondary Use of Human Tissue", Federation of Medical Scientific Societies). For the comparison of LINE-1 methylation levels in tumor and adjacent normal epithelium, for 28 patients, normal tissue was also collected.

## DNA EXTRACTION FROM FORMALIN-FIXED PARAFFIN-EMBEDDED TUMOR TISSUES AND BISULFITE CONVERSION



In order to reduce the amount of tumor stromal tissue components, tumor areas (>80% neoplastic epithelial cells) on haematoxylin and eosin-stained tumor sections were marked. Of each patient block, five FFPE tumor tissue sections of 7 μm were deparaffinized and stained with haematoxylin, followed by manual needle microdissection of the marked areas. After microdissection, genomic DNA was extracted from the collected tumor material using the Microlab starLET IVD robot (Hamilton Robotics, Bonaduz, Switzerland) and quantified using a Qubit® 2.0 Fluorometer according to the Qubit ds DNA HS Assay kit protocol (Invitrogen™, Eugene, OR, USA). Bisulfite conversion was performed with EZ DNA Methylation Gold kit (Zymo Research Corp, Orange, CA, USA) on the isolated genomic DNA, according to the manufacturer's instructions, using an input of 50 ng of DNA. Bisulfite converted DNA was eluted in 15 μL Milli-Q purified water.

### QUANTITATIVE REAL-TIME PCR

The PCR primers used in this study amplified the target sequence independent from methylation status. In addition, minor-groove-binding (MGB) methylation specific probes were used as previously used by Sunami et al.[12]. Primer sequences were as follow; 5′-GGGTTTATTTTATTAGGGAGTGTTAGA-3′ (forward), 5′-TCACCCCTTTCTTTAACTCAAA-3′ (reverse). The probes were labelled with Hexachloro-Fluorescein (HEX) and 6-fluorescein amidite (FAM) sequences were as follows; Allele 1-FAM-5′-TGCGCGAGTCGAAGT-3′-MGB-BHQ (methylated-specific) and Allele 2-HEX-5′-TGTGTG AGTTGAAGTAGGG-3′-MGB-BHQ (unmethylated-specific) (Biolegio, Nijmegen, the Netherlands). Real-time PCR was performed in a final reaction volume of 10 µL consisting of 1 µl bisulfite converted DNA template, 200 µM deoxynucleotide triphosphates (dNTPs), 1.9 µM $MgCl_2$, 1x PCR Gold Buffer, 1 unit of AmpliTaq Gold DNA Polymerase® (Applied Biosystems, Foster City, CA, USA), 0,4 µM of the forward and reverse primer, 0,25 µM of MGB probes. The following protocol was executed: 5min 95°C, 7x (15sec 95°C, 30sec 64°C with a 1°C decrement every cycle, 20sec 72°C); 39x (15sec 95°C, 30sec 52°C, 20sec 72°C); 1min 60°C and a melt curve from 65°C to 95°C with a increment of 0.5°C for 10sec. Quantitative PCR reactions were run on a CFX96™ Real-Time system C1000™ Thermal cycler (BioRad, Benicia, CA, USA). All reactions were performed in triplicate. Controls used for LINE-1 methylation assays were universally methylated DNA (Millipore, Billerica, Massachusetts, USA) and universally unmethylated DNA obtained by repeated whole genome amplification of peripheral blood lymphocyte DNA (Repli-g kit Qiagen, Valencia, CA, USA). A standard curve was generated by the use of a mix with proportions (0%,10%,25%,40%,60%,75%,90% and 100%) of the universally methylated and unmethylated DNA and was used for quantification of LINE-1 methylation levels in the patient samples.

### ANALYSIS OF LINE-1 METHYLATION LEVELS

Relative fluorescence units (RFU) were used to calculate the level of methylated DNA. Samples with higher quantities of amplified methylated or unmethylated DNA have higher RFU values for Allele 1-FAM or Allele 2-HEX respectively. A standard curve was generated with the RFU of the Allele 1-FAM-probe and Allele 2-HEX-probe which were obtained by using a mixture of universally methylated and unmethylated DNA in different ratios as indicated in the previous paragraph. The RFU for both probes were plotted followed by the calculation of the angle (α) (Supplementary figure S1A), as a measure of the ratio between methylated-unmethylated LINE-1 elements. For each sample the mean α of the triplicates was calculated. By using the standard curve, the LINE-1 methylation level was determined by looking up the corresponding angle (α).

4.5. MLH1 methylation status

MLH1 methylation analyses were performed using the bisulfite converted tumor DNA (described above) according to the protocols developed at the molecular diagnostics of the department of Pathology at the LUMC as described by van Roon et al. [39].

### STATISTICAL ANALYSIS

Statistical analyses were performed using the statistical package SPSS (version 20.0 for Windows; SPSS Inc.). Student's T test and the Chi-squared test were used for the evaluation of the association between LINE-1 methylation levels and clinical-pathological parameters. Overall survival (OS) was defined as time of surgery until death. Disease-free survival (DFS) was defined as time of surgery until relapse of the disease or death, whichever came first. The definition of recurrence-free survival (RFS) was time of surgery until local or distant recurrences, whichever came first. Deaths were censored in this analysis. For survival probabilities the Kaplan-Meier method was used, for comparison of the survival curves the Log-Rank test was used. Kaplan-Meier curves were censored at 10 years of follow-up. Univariate and multivariate Cox regression analyses were performed to evaluate the differences in OS, DFS and RFS in patients with methylated versus patients with hypomethylated LINE-1 elements. The tumor LINE-1 hypomethylated group consisted of patients with the lowest one third of the calculated methylation levels. The tumor LINE-1 methylated group consisted of patients with the upper two third of the methylation levels. To investigate differential association of LINE-1 methylation level in survival by molecular subtype and age, pre-specified stratified analyses were performed for MLH1 methylation status and age followed by interaction analysis which was assessed with the Wald test. Covariates entered in the multivariate model were age, sex, and MLH1 methylation status. For all tests, a P-value of <0.05 was considered as statistically significant.



## RESULTS

### PATIENT CHARACTERISTICS

In total, 181 patients were included based on the availability of FFPE tissue blocks. After manual needle microdissection, a sufficient amount of tumor tissue was collected and successful DNA extraction was performed for 164 out of 181 patients. Table 1 summarizes the clinical, pathological and treatment characteristics of the patients included for analyses (n=164).

## METHYLATION ASSAY VERIFICATION

To ensure reproducibility and a good performance of the LINE-1 methylation assay, several quality checks were performed. The inter assay variation of the control samples, showed minimal inter-plate variations: (<7%). A standard curve, using proportions of the universally methylated and unmethylated DNA was constructed and showed to be highly reproducible, with $r^2 \geq 0.96$ (Supplementary figure S1B). To control variation, each DNA sample was run in triplicate. As indicated in Supplementary figure S1C, the three angles (α) of each patient sample were highly comparable.

**Table 1:** Baseline clinical and pathological characteristics for the total cohort (n=164) and stratified for tumor LINE-1 methylation status. Data are presented as n (%) or as median ±SD.

| | | | LINE-1 | | | | |
|---|---|---|---|---|---|---|---|
| | Total | | Hypomethylated[a] | | Methylated[b] | | P-value |
| | *n*=164 | (%) | *n*=54 | (%) | *n*=110 | (%) | |
| Gender | | | | | | | |
| Male | 86 | (52.4) | 30 | (55.6) | 56 | (65.1) | 0.58 |
| Female | 78 | (47.6) | 24 | (44.4) | 54 | (49.1) | |
| Age median | 68.0 | (±13.6) | 69.5 | (±12.0) | 68.0 | (14.3±) | 0.16 |
| Age groups | | | | | | | |
| ≤ 65 | 66 | (40.2) | 17 | (31.5) | 49 | (44.5) | 0.11 |
| >65 | 98 | (59.8) | 37 | (68.5) | 61 | (55.5) | |
| Grade | | | | | | | |
| Good | 32 | (19.5) | 11 | (20.4) | 21 | (19.1) | 0.24 |
| Moderate | 79 | (48.2) | 30 | (55.6) | 49 | (48.2) | |
| Poor | 20 | (12.2) | 7 | (13.0) | 13 | (11.8) | |
| Unknown | 33 | (9.8) | 6 | (11.1) | 27 | (24.5) | |
| Location | | | | | | | |
| Right | 74 | (45.1) | 22 | (40.7) | 52 | (47.3) | 0.43 |
| Left | 90 | (54.9) | 32 | (59.3) | 58 | (52.7) | |
| *MLH1* promoter | | | | | | | |
| Methylated | 30 | (18.3) | 6 | (11.1) | 24 | (21.8) | 0.10 |
| Unmethylated | 134 | (81.7) | 48 | (88.9) | 86 | (78.2) | |
| Adjuvant therapy | | | | | | | |
| No | 156 | (95.1) | 50 | (92.6) | 106 | (96.4) | 0.29 |
| Yes | 8 | (4.9) | 4 | (7.4) | 4 | (3.6) | |

Data are presented as median ± SD or n(%)

[a] Hypomethylated group includes 1/3 of the patient cohort with the lowest methylation levels

[b] Methylated group includes 2/3 of the patient cohort with higher methylation levels

## LINE-1 METHYLATION LEVEL AND PATIENT SURVIVAL

As shown in Figure 1, normal colon tissues (n=28) showed significantly different relative LINE-1 methylation levels (58.2% ±SD 14.9%) compared with tumor tissue of the total patient cohort of 164 patients (40.7% ±SD 17.1%) with $P<0.001$.

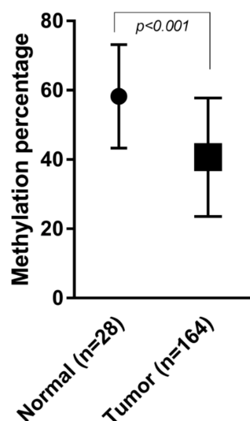


**Figure 1**: Average LINE-1 methylation level in normal and tumor epithelium. Mean methylation level of 28 normal colon epithelial tissues (58.2% ±SD 14.9%) compared with 164 stage II colon tumor tissue samples (40.7% ±SD 17.7%). Error bars represent the standard deviation of the mean.

Based on the calculated methylation levels of tumor DNA, a methylated and a hypomethylated group were defined for comparison. The tumor LINE-1 hypomethylated group consisted of patients with the lowest one third of the calculated methylation levels (median relative LINE-1 methylation level; 24.1%). The tumor LINE-1 methylated group consisted of patients with the upper two third of the methylation levels (median relative LINE-1 methylation level; 45.3%). The mentioned percentages are not reflecting the absolute percentages, but a relative measure in comparison to the used standards (Supplementary figure 1.)

No significant differences were observed between the hypomethylated LINE-1 group (n=54) and methylated LINE-1 group (n=110), regarding the clinical and pathological characteristics (Table 1). As shown in the Kaplan-Meier survival curve (Figure 2), patients with hypomethylated LINE-1 tumors had a significantly worse OS compared with patients in the methylated LINE-1 group with a significant Log-Rank test. The Cox regression model for overall survival showed a hazard ratio (HR) of 1.75 (95%CI 1.09-2.81; *P*=0.02, Table 2). Multivariate analysis, adjusted for age, gender and *MLH1* methylation status of the OS showed a HR of 1.68 (95%CI 1.03-2.75; *P*=0.04, Table 2). No statistically significant difference for disease-free survival (DFS) was observed in the Kaplan-Meier survival curve (Figure 2), univariate (HR 1.52, 95%CI 0.97-2.40; *P*=0.07) and multivariate analysis (HR 1.43, 95%CI 0.90-2.29; *P*=0.13, Table 2). Furthermore, no significant difference between patients in the hypomethylated and methylated group was found for relapse-free survival (RFS) in univariate and multivariate analyses (Table 2).

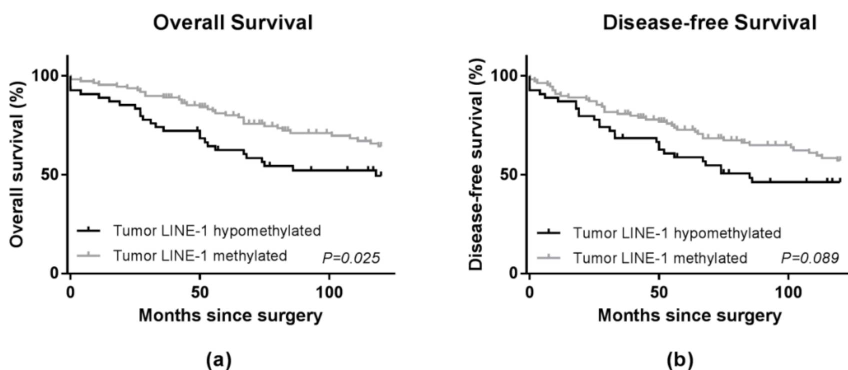


**Figure 2**: Survival curves for overall survival (**a**) and disease-free survival (**b**) in 164 patients with stage II colon cancer. Solid black line represents patients with tumor LINE-1 hypomethylation. The grey line represents patients without tumor LINE-1 hypomethylation. The *P*-value in the graphs represents the Log-Rank value.

**Table 2:** Univariate and multivariate analyses for overall survival, disease-free survival and recurrence-free survival comparing tumor LINE-1 methylated and tumor LINE-1 hypomethylated, in stage II colon cancer in the total cohort and stratified by *MLH1* status.

| | Patients | Univariate | | Multivariate[a] | |
|---|---|---|---|---|---|
| | *n*=164 | HR (95%CI) | P-value | HR (95%CI) | P-value |
| **Overall Survival** | | | | | |
| LINE-1 Methylated | 110 | 1.00 (*reference*) | 0.02 | 1.00 (*reference*) | 0.04 |
| LINE-1 Hypomethylated | 54 | 1.75 (1.09-2.81) | | 1.68 (1.03-2.75) | |
| **Disease-free Survival[b]** | | | | | |
| LINE-1 Methylated | 110 | 1.00 (*reference*) | 0.07 | 1.00 (*reference*) | 0.13 |
| LINE-1 Hypomethylated | 54 | 1.52 (0.97-2.40) | | 1.43 (0.90-2.29) | |
| **Recurrence-free Survival[c]** | | | | | |
| LINE-1 Methylated | 110 | 1.00 (*reference*) | 0.37 | 1.00 (*reference)* | 0.33 |
| LINE-1 Hypomethylated | 54 | 1.40 (0.66-2.97) | | 1.46 (0.68-3.15) | |
| **Overall Survival by *MLH1* status** | | | | | |
| *MLH1* unmethylated | 134 | | | | |
| LINE-1 Methylated | 86 | 1.00 (*reference*) | 0.02 | 1.00 (*reference*) | 0.09 |
| LINE-1 Hypomethylated | 48 | 1.85 (1.10-3.11) | | 1.56 (0.93-2.63) | |
| *MLH1* methylated | 30 | | | | |
| LINE-1 Methylated | 24 | 1.00 (*reference*) | 0.89 | - | - |
| LINE-1 Hypomethylated | 6 | 1.09 (0.29-4.07) | | - | |

[a]Adjusted for age, gender, MLH-1 methylation status. [b]Disease-free survival defined as time from operation until local-regional recurrence, distant recurrence or death whichever came first. [c]Recurrence- free period define as time from operation until any recurrence (local-regional recurrence and/or distant recurrence) whichever came first.

Analyses for tumor LINE-1 hypomethylation stratified by age, ≤65 years at operation and >65 years at operation, were performed since global hypomethylation was reported to be associated with increasing age [19]. As shown in Figure 3 and Table 3, a significantly worse OS for patients with hypomethylated LINE-1 tumors was observed in the patients older than 65 years at operation in the univariate (HR 1.93, 95%CI 1.15-3.26; *P*=0.01) and multivariate analysis (HR 2.00, 95%CI 1.13-3.52; *P*=0.02). Furthermore, in multivariate analysis of DFS, a significant unfavorable prognosis was found for patients older than 65 with LINE-1 hypomethylated tumors (HR 1.76, 95%CI 1.02-3.05; *P*=0.04). No significant differences were found in RFS (Table 3). In contrast, in patients with colon cancer who were younger than 65 years of age at time of diagnosis, no significant differences in OS, DFS or RFS were observed regarding LINE-1 methylation levels (Table 3). The Wald tests showed non-significant p-values for interaction (Table 3), which suggests that the difference found between the two age groups could be based on chance.

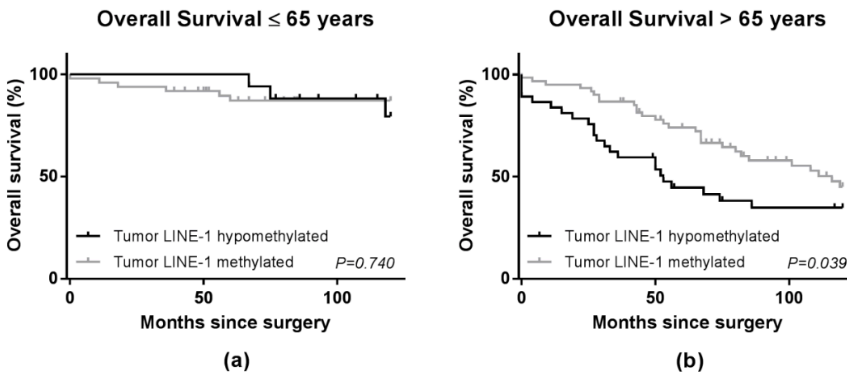


**Figure 3:** Survival curves for overall survival in colon cancer stage II patients stratified by age in two groups; ≤65 years at operations (n=66) (**a**) and >65 years at operation (n=98) (**b**). Solid black line represents patients with tumor LINE-1 hypomethylation. The grey line represents patients without tumor LINE-1 hypomethylation. The *P*-value in the graphs represents the Log-Rank value.

*MLH1* promoter hypermethylation was found in 18.3% of the patients. *MLH1* promoter methylation is the most commonly observed alteration causing MSI in sporadic colon tumors [23], and consequently these tumors with MLH1 promoter methylation are likely to represent most of the MSI tumors the cohort, although we did not evaluate MSI independently. The effect of tumor LINE-1 methylation was analyzed in patients with and without *MLH1* methylated promoters separately. Corresponding with the total study cohort, an unfavorable clinical outcome was observed in patients with LINE-1

hypomethylated tumors (HR 1.85, 95%CI 1.10-3.11; *P*=0.02) within the patients without *MLH1* promoter hypermethylation (Table 2). Multivariate analysis of the OS in this group showed a HR of 1.56 (95%CI 0.93-2.63; *P*=0.09, Table 2). LINE-1 methylation did not correlate with survival in patients with *MLH1* promoter hypermethylation. Due to the low number of patients with methylated *MLH1* promoters (n=30), no multivariate analysis was performed (Table 2).

**Table 3:** Hazard Ratio for overall survival, disease-free survival and recurrence-free survival comparing methylated LINE-1 and hypomethylated LINE-1 stage II colon tumors stratified by age. Hazard ratios are displayed for both Cox proportional hazard univariate and multivariate analysis with 95% confidence interval. The Wald test was performed to calculated the *P*-value for interaction.

| | Patients n=164 | Univariate | | Multivariate[a] | | P-value for interaction[b] |
|---|---|---|---|---|---|---|
| | | HR (95%CI) | P-value | HR (95%CI) | P-value | |
| **Overall Survival** | | | | | | |
| ≤65 years | 66 | | | | | |
| LINE-1 Methylated | 49 | 1.00 (*reference*) | 0.82 | 1.00 (*reference*) | 0.80 | |
| LINE-1 Hypomethylated | 17 | 0.81 (0.22-3.04) | | 0.84 (0.23-3.16) | | 0.24 |
| >65 years | 98 | | | | | |
| LINE-1 Methylated | 61 | 1.00 (*reference*) | 0.01 | 1.00 (*reference*) | 0.02 | |
| LINE-1 Hypomethylated | 37 | 1.93 (1.15-3.26) | | 2.00 (1.13-3.52) | | |
| **Disease-free Survival[c]** | | | | | | |
| ≤65 years | 66 | | | | | |
| LINE-1 Methylated | 49 | 1.00 (*reference*) | 0.76 | 1.00 (*reference*) | 0.71 | |
| LINE-1 Hypomethylated | 17 | 0.84 (0.27-2.56) | | 0.71 (0.26-2.53) | | 0.31 |
| >65 years | 98 | | | | | |
| LINE-1 Methylated | 61 | 1.00 (*reference*) | 0.05 | 1.00 (*reference*) | 0.04 | |
| LINE-1 Hypomethylated | 37 | 1.67 (1.00-2.77) | | 1.76 (1.02-3.05) | | |
| **Recurrence-free Survival** | | | | | | |
| ≤65 years | 66 | | | | | |
| LINE-1 Methylated | 49 | 1.00 (*reference*) | 0.93 | 1.00 (*reference*) | 0.99 | |
| LINE-1 Hypomethylated | 17 | 0.94 (0.25-3.55) | | 1.00 (0.26-3.08) | | 0.35 |
| >65 years | 98 | | | | | |
| LINE-1 Methylated | 61 | 1.00 (*reference*) | 0.28 | 1.00 (*reference*) | 0.11 | |
| LINE-1 Hypomethylated | 37 | 1.67 (0.66-4.23) | | 2.32 (0.83-6.52) | | |

[a]Adjusted for gender, MLH-1 methylation status. [b]P-value for interaction was calculated for univariate analysis. [c]Disease-free survival defined as time from operation until local-regional recurrence, distant recurrence or death, whichever came first [d]Recurrence- free period define as time from operation until any recurrence (local-regional recurrence and/or distant recurrence), whichever came first.

# DISCUSSION

Genome-wide DNA hypomethylation is an important epigenetic alteration in CRC [2,3]. Furthermore, it has been proposed that loss of global DNA methylation is strongly associated with hypomethylated LINE-1 elements, subsequently LINE-1 methylation serves as a surrogate marker for overall DNA methylation status [4]. Accurate identification and isolation of tumor cells is highly important in studying tumor LINE-1 methylation, considering tumor-associated stromal cells could influence the measured tumor methylation levels and consequently bias the results. A study conducted by Irahara *et al*. demonstrated that results regarding LINE-1 methylation levels obtained with manual needle microdissection were comparable to isolating tumor cells by laser capture technique [24]. Accordingly, needle microdissection of tumor cells was applied in this study, consequently a minimal contaminating effect of DNA from stromal cells could be expected.

Many studies have reported on the effect of LINE-1 hypomethylation, showing that tumor LINE-1 hypomethylation results in a worse prognosis in CRC patients, especially in patients with proximal located colon tumors [10-12,14,25]. Interestingly, the majority of the performed studies did not reveal a relation between LINE-1 hypomethylation and clinical outcome in more advanced disease stages [12-14]. For that reason, only patients with stage II colon cancer were included in this study to evaluate the assumed prognostic value of LINE-1 hypomethylation in early-stage colon cancer without the interference of patients with advanced disease stages or patients with rectal cancers. In line with the above-mentioned studies, we observed a significantly worse overall survival in patients with stage II colon tumors with LINE-1 hypomethylation, compared to those with tumors with higher LINE-1 methylation levels, supporting the role of LINE-1 as a prognostic biomarker. In contrast, no significant differences were observed in DFS and RFS among patients with hypomethylated and methylated tumors. Based on the results obtained in this study, a specific role for LINE-1 hypomethylation as a biomarker, for colon cancer disease progression could not be suggested. Combined with the fact that no correlation between LINE-1 hypomethylation and survival was observed in more advanced disease stages, global loss of DNA methylation appears more as an early event in colon cancer formation rather than during disease progression [26-28]. The contribution of LINE-1 hypomethylation in colon cancer formation is supported by the study of Pavicic *et al.*, in which LINE-1 methylation levels were evaluated in normal epithelial tissues of patients with hereditary nonpolyposis colorectal cancer (HNPCC), familiar colorectal cancer and sporadic cancer [29]. They found the lowest LINE-1 methylation levels in normal mucosa of patients with familial CRC, suggesting that lower levels of LINE-1 methylation predispose normal tissue to cancer development. Furthermore, patients diagnosed with serrated

polyps (at risk of synchronous CRC development) did show LINE-1 hypomethylation in the normal adjacent colon mucosa [30]. Moreover, studies revealed that colon mucosa shows an age-related global hypomethylation [19,28]. Models have been developed to predict chronological age from DNA methylation [31]. A number of studies indicate that a discrepancy between chronological age and age predicted based on DNA methylation patterns (i.e., predicted age based on methylation exceeding chronological age), has been associated with an increased mortality risk [32,33]. This could explain the observed worse OS, without differences in DFS and RFS, in patients with LINE-1 hypomethylated tumors, especially in patients aged over 65 years. Accordingly, LINE-1 hypomethylation might be associated with an increased mortality risk in general, rather than a more aggressive tumor. This suggests that LINE-1 hypomethylation does not contribute to disease progression, although a prognostic role for LINE-1 hypomethylation in a more general way could be considered. Notably, no statistical interaction between the age groups was observed; the effect of age on the association of LINE-1 methylation status and survival could be based on chance. Therefore, conclusions on LINE-1 hypomethylation in combination with age have to be drawn carefully.

Conflicting results have been published regarding tumor LINE-1 hypomethylation in combination with MSI status in CRC in relation to survival [21,22]. *MLH1* promoter methylation is the most commonly observed alteration causing MSI in sporadic colon tumors [23]. In the general population 15% of the sporadic colon tumors are MSI [20,34-36], consistent with the observed 18.5% of the tumors in our cohort. Unfortunately, the number of patients in this subgroup was too small to draw a conclusion on the effect of LINE-1 hypomethylation in patients with MSI colon tumors. Larger study cohorts will be needed to firmly analyze MSI in combination with LINE-1 methylation levels.

Tumor LINE-1 hypomethylation, was used as surrogate marker for global DNA hypomethylation in this study. Based on the results of this study, a role for global DNA hypomethylation in colon cancer development, rather than LINE-1 methylation level as a biomarker for disease progression, could be suggested. Additional studies to further evaluate, whether or not LINE-1 hypomethylation has a specific role in disease progression in stage II colon cancer will be needed. Large cohorts will be essential, considering the low tumor recurrence rates in patients with early-stage colon cancer. Besides a surrogate marker for genome-wide hypomethylation, LINE-1 hypomethylation could result in increased retrotransposition activity and integration of LINE-1 elements near oncogenes or tumor suppressor genes and may influence cancer development or disease progression [37,38]. Further research will be essential to fully unravel these complex mechanisms in the scope of colon cancer development and disease progression.

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# Chapter 9

## No effect of microsatellite status on outcome of rectal cancer

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*In preparation*

## ABSTRACT

Currently, compelling evidence illustrates the significance of determining microsatellite instability (MSI) in colorectal cancer (CRC). The association of MSI with proximal CRC is well established, however, its implications in patients with rectal cancer remain undefined. We therefore aimed to determine the role of MSI in respect to incidence and outcome in patients with rectal cancer, by the examination of patients from two prospective phase III trials: TME trial and PROCTOR-SCRIPT trial (n=1250). No significant differences in terms of overall survival (HR 1.00, 95%CI 0.69-1.47) and disease-free survival (HR 1.00, 95%CI 0.68-1.45) were observed in patients with MSI or MSS rectal cancer. In addition we performed a literature review to evaluate the overall prevalence, the effect on survival and the response to neo-adjuvant treatment in patients with MSI rectal cancer compared with MSS rectal cancer. The total number of MSI cases in the included studies (including our own) was 317 (out of 5448 rectal cancer patients), with an overall prevalence of 5.8% (SE 2.3%). Both for overall survival as for disease-free survival there was no impact of MSI status on prognosis (HR 1.02, 95%CI 0.75-1.38 and HR 1.09, 95%CI 0.79-1.50, respectively. The risk ratio for response on neoadjuvant chemoradiation showed heterogeneity ($I^2$ = 68%) and included 38 cases with MSI with an overall risk ratio of 0.9 (95%CI 0.36-2.24). In conclusion, rectal cancer patients with MSI form a distinct and rare subcategory of CRC, however, there is no prognostic effect of MSI in rectal cancer patients.


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# INTRODUCTION

Microsatellite instability (MSI) is one of the most established biomarkers in CRC. MSI represents a hallmark of Lynch syndrome, however the majority of MSI tumors are found in sporadic CRC. Approximately 15% of the sporadic stage II-III CRC has MSI [1]. MSI -CRC have distinct features such as a more proximal localization, higher grade, a mucinous histology with tumor infiltrating lymphocytes and in the sporadic setting the presence of a BRAF mutation [2,3]. The relation of MSI outcome is complex: in early stage CRC it is associated with a prognostic advantage [4-7]. In contrast, in metastatic disease MSI is associated with a poor clinical outcome [8]. Although with conflicting results, accumulating preclinical and clinical evidence reports a resistance to 5-fluorouracil (5-FU) based chemotherapy, in CRC patients with MSI tumors [5-7,9]. Currently, compelling evidence illustrates the significance of determining MSI in CRC. 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) [10-12].

The implications of MSI in patients with rectal cancer are still undefined. Due to the well documented differences between proximal and distal CRC with respect to prognosis, molecular background and treatment [13-15], it is clear that known implications of MSI (mainly obtained from patients with proximal CRC) cannot be extrapolated to patients with rectal cancer specifically [16,17]. Consequently, there is a clinical urgency to determine the impact of MSI in rectal cancer patients. Based on *in vitro* experiments and in small patient series, an altered radiosensitivity in MSI tumors has been suggested [18,19]. Charara *et al,* suggested that rectal cancer patients with MSI tumors may have increased responses rates [20].

We therefore aimed to determine the role of MSI in respect to incidence and outcome in patients with rectal cancer, by the examination of patients from two prospective phase III trials: TME trial and PROCTOR-SCRIPT trial.



# MATERIALS AND METHODS

## PATIENT SELECTION

Data were derived from patients with rectal cancer included in the Dutch TME trial (*n*=1530) and the PROCTOR-SCRIPT trial (*n*=470), of which the results have been published previously [16,17]. Informed consent for participation and retrospective use of

samples was obtained from all patients enrolled in both trials. All cases were considered as sporadic rectal cancer, based on the inclusion criteria of both trials. Formalin-fixed paraffin-embedded (FFPE) tissue of the included Dutch patients were collected. As shown in figure 1, sufficient FFPE tumor material was available for n=1061 patients of the TME study. In the PROCTOR-SCRIPT study, *n*=324 Dutch patients were included, tumor tissue could be obtained of n=268 patients, resulting in a total study cohort of *n*=1329 patients with rectal cancer. Histopathological representative tumor regions on hematoxylin and eosin (HE) stained tumor sections were marked by a pathologist (AvT) and punched for the preparation of tumor tissue microarrays (TMA).

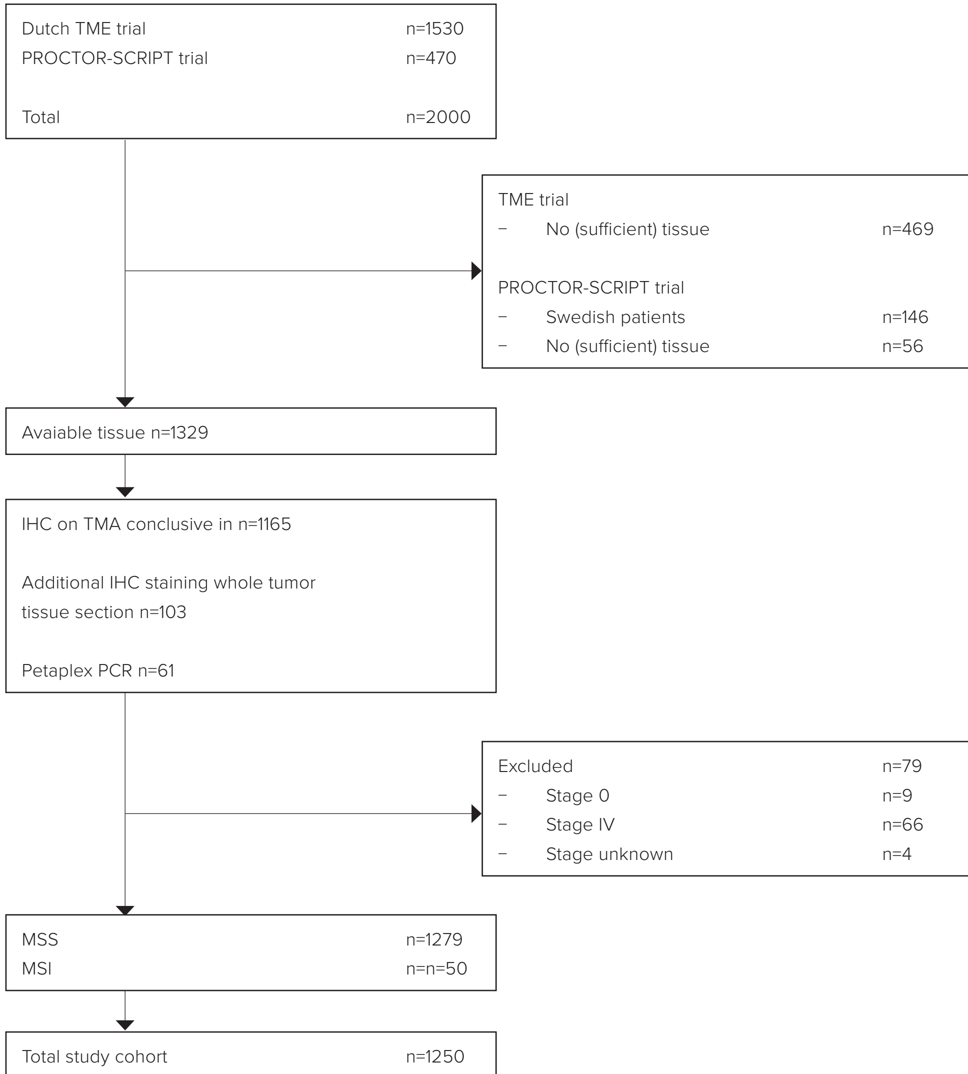



**Figure 1.** Patient selection.

### MICROSATELLITE ANALYSIS BY IMMUNOHISTOCHEMISTRY

Immunohistochemical staining for MMR proteins was performed on 4μm TMA sections, with antibodies against MLH1, PMS2, MSH2 and MSH6. Briefly, TMA sections underwent deparaffinization and rehydration using xylene and a graded ethanol into water series. Heat-induced antigen retrieval was performed in EDTA for 10 minutes . Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 minutes at room temperature. Sections were incubated in predetermined optimal dilutions (MLH1 1:40, PMS2 1:100, MSH2 1:40, MSH6 1:500), for 60 minutes at room temperature with anti-MLH1 (clone G168-15, mouse, BD Biosciences, San Jose, California, United States), anti-PMS2 (clone A16-4, mouse, BD Biosciences, San Jose, California, United States), anti-MSH2 (clone GB12, mouse, Calbiochem/Merck, Darmstadt, Germany) and anti-MSH6 (clone EPR3945, rabbit, Abcam, Cambridge, United Kingdom). Sections were incubated with Brightvision+poly-HRP-anti Ms/Rb/Rt IgG (Immunologic, Duiven, the Netherlands) for 30 minutes by room temperature, followed by 7 minutes incubation with 3,3’-diaminobenzidine (DAB, immunologic, Duiven, the Netherlands) to visualize antigen expression. Sections were counterstained with haematoxylin, dehydrated and coverslipped. Tissue stroma served as internal positive control for the staining with anti-MLH1, anti-PMS2, anti-MSH2 and anti-MSH6.

Microscopic analysis of MLH1, PMS2, MSH2 and MSH6 expression was performed by two independent observers (AvT and MS) in a blinded manner. When MMR protein expression obtained with IHC on a TMA was inconclusive, additional PCR analysis was performed, as described below.

### DNA EXTRACTION AND PENTAPLEX PCR ANALYSI*s*

Areas containing tumor cells were selected by microscopic evaluation on a slide stained with H&E by a pathologist (AvT). DNA was extracted from manual microdissected sections of FFPE tissue by incubation in 5% Chelex-100 in TET lysisbuffer and 10% Proteinase K (20mg/ml) (Qiagen, Hilden, Germany) for 16 hours at 56°C. MSI analysis was performed using five mononucleotide repeat markers (NR-21, NR-24, NR-27, BAT-25 and BAT-26) in a single multiplex PCR [21]. The PCR was carried out on a MJ Research PTC-200 Thermal Cycler™ using 5PRIME HotMaster Taq DNA polymerase® (QuantaBio, Beverly, United States) with 1μl DNA and the following program; initial denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 10 s, and extension at 65°C for 30 s, with a final extension at 65 °C for 7 min. DNA fragment analysis was executed on the 3730 DNA Analyzer (Applied Biosystems, Foster City, California, United States). Product sizes for the markers were determined using GeneMarker V.2.6.7 (Applied Biosystems). Normal colon tissues were used as control. A tumor was defined as MSI if at least two of the five markers showed instability.

## STATISTICAL ANALYSIS

Statistical analyses were performed using the statistical package SPSS (version 20.0 for Windows; SPSS Inc.). Student's T test and the Chi-squared test were used for the evaluation of the association between MSI and MSS and clinical-pathological parameters. Overall survival (OS) was defined as time of surgery until death. Disease-free survival was defined as time to any recurrence or death, whichever occurred first, or end of follow-up (censored). Distant recurrence (DR) and locoregional recurrence (LRR) were defined as time of surgery until distant recurrence and locoregional recurrence respectively. Deaths were censored in this analysis. For survival probabilities the Kaplan-Meier method was used and for comparison of survival curves the Log-Rank test were used. Univariate and multivariate Cox regression analyses were performed to evaluate the differences in OS, DR, and LRR. Covariates entered in the multivariate model were age, disease stage, pre-operative treatment and adjuvant treatment. For all tests a *p*-value of <0.05 was considered as statistical significant.

## REVIEW OF LITERATURE

In cooperation with a trained librarian, we searched for published research comparing patients with MSI rectal cancer and MSS rectal cancer. We searched for "rectal neoplasms" and "microsatellite instability" as search terms in Pubmed, including all relevant keyword variations. Titles and abstracts were screened of retrieved articles followed by full-text review of studies that seemed to evaluate MSI/MSS status in rectal cancer patients in relation to clinical outcome.

For each study the number of patients in both the MSI and the MSS group were obtained. Data on response rate, 5-year DFS, and 5-year OS were extracted from all studies. If no HR was reported, it was calculated from Kaplan-Meier curves [22]. Data were entered in Review Manager (RevMan). Version 5.3. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2012). A meta-analysis was performed with all available studies on each endpoint in terms of risk ratios (RR) and hazard ratio (HR) with 95% confidence interval (CI). A random effects model with inverse variance weighting of studies was used. Heterogeneity was assessed using a $\chi^2$ test for heterogeneity with a *p*-value of <0.10 to show the presence of significant heterogeneity.

# RESULTS

## STUDY POPULATION

In total, tumor tissue from 1329 patients could be obtained and was suitable for the preparation of a TMA. Of the total study cohort 1061 patients participated in the TME trial and 268 patients in the PROCTOR-SCRIPT trial. Patients with tumor stage 0, stage IV or unknown tumor stage were excluded (*n*= 79). In total 1250 patients were included for analysis, with a median follow up of 7.4 years. Of the included patients 503 patients underwent TME surgery without neoadjuvant treatment, 718 patients received neoadjuvant radiotherapy and 28 patients received neoadjuvant chemoradiation. In the total patient cohort (n=1250) MSI has been observed in 48 (3.8%) and 1202 (96.2%) tumors were considered MSS. The patient and tumor characteristics of the total cohort and stratified by MSS or MSI status were summarized in table 1. No significant differences were observed between patients with MSI tumors and MSS tumors regarding clinicopathological characteristics.

**Table 1.** Patient characteristics of the total study cohort and stratified for MSI and MSS status.

| | **Total** *n*=1250 | | **MSI** *n*=48 | | **MSS** *n*=1202 | | **p-value** |
|---|---|---|---|---|---|---|---|
| | *n* | (%) | *n* | (%) | *n* | (%) | |
| Gender | 797 | (63.8) | 30 | (62.5) | 767 | (63.8) | 0.88 |
| Male | 453 | (36.2) | 18 | (37.5) | 435 | (36.2) | |
| Female | | | | | | | |
| Age median | 64.0 | (±10.9) | 62.0 | (±11.5) | 64.0 | (±10.8) | 0.10 |
| Disease stage | | | | | | | |
| I | 325 | (26.0) | 10 | (20.8) | 315 | (26.2) | 0.53 |
| II | 337 | (27.0) | 16 | (33.3) | 321 | (26.7) | |
| III | 588 | (47.0) | 22 | (45.8) | 566 | (47.1) | |
| Neoadjuvant treatment | | | 18 | | | | 0.98 |
| None | 503 | (40.2) | 29 | (37.5) | 485 | (40.3) | |
| Radiotherapy | 718 | (57.4) | 1 | (60.4) | 689 | (57.3) | |
| Chemoradiotherapy | 28 | (2.2) | 0 | (2.1) | 27 | (2.2) | |
| Other | 1 | (0.1) | | (0) | 1 | (0.1) | |
| Adjuvant treatment | | | | | | | 0.36 |
| Observation | 1022 | (81.7) | 41 | (85.4) | 980 | (81.5) | |
| Chemotherapy | 177 | (14.1) | 6 | (12.5) | 171 | (14.2) | |
| Radiotherapy | 43 | (3.4) | 0 | (0) | 43 | (3.6) | |
| Other | 9 | (0.7) | 1 | (2.1) | 8 | (0.7) | |
| CRM | | | | | | | |
| Negative | 1066 | (85.2) | 38 | (79.2) | 1027 | (85.4) | 0.40 |
| Positive | 180 | (14.4) | 10 | (20.8) | 170 | (14.1) | |
| Unknown | 5 | (0.4) | 0 | (0) | 5 | (0.4) | |

*Data are presented as median ± SD or n(%)*

### SURVIVAL DATA

As shown in figure 2 and table 2, no significant differences in terms of OS (HR 1.00, 95%CI 0.69-1.47), DFS (HR 1.00, 95%CI 0.68-1.45), DR (HR 0.94, 95%CI 0.54-1.63) and LRR (HR 1.52, 95%CI 0.62-3.75) were observed in patients with MSI or MSS rectal cancer. In the multivariate analysis, no significant difference were observed (table 2).

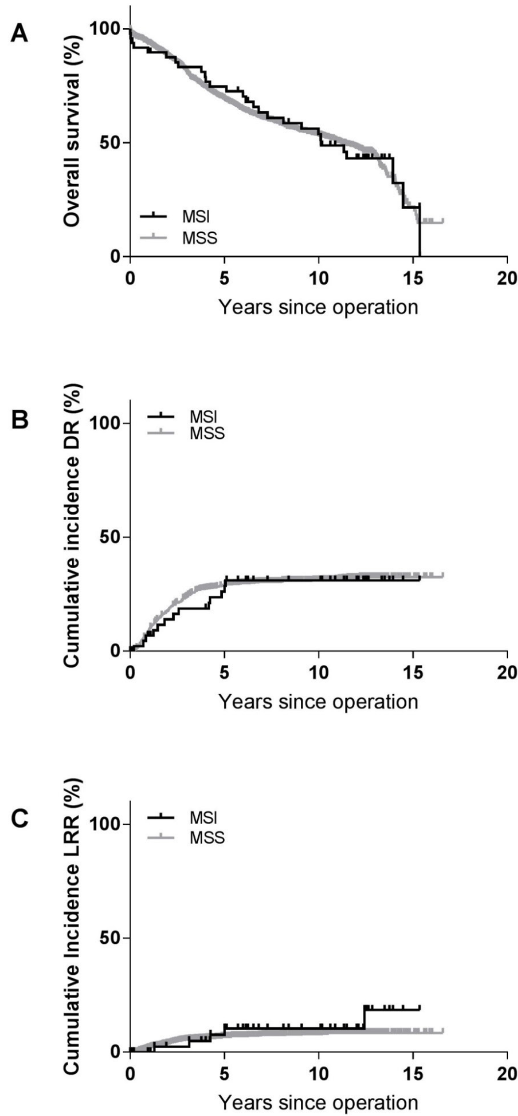



**Figure 2.** Kaplan-Meier analysis for overall survival (A), time to distant recurrence (B) en time to local recurrence (C) in 1250 rectal cancer patients according to MSS or MSI (n=48) status.

**Table 2.** Univariate and multivariate survival analysis for overall survival, disease-free survival, time to distant recurrence and time to local recurrence according to MSI and MSS status. Covariates entered in the multivariate model were age, neoadjuvant treatment, adjuvant treatment, disease stage.

| | Patients n=1250 | Univariate | | Multivariate | |
|---|---|---|---|---|---|
| | | HR (95%CI) | P-value | HR[a] (95%CI) | P-value |
| **Overall Survival** | | | | | |
| MSI | 48 | 1.00 (0.69-1.47) | 0.99 | 1.20 (0.82-1.76) | 0.35 |
| MSS | 1202 | 1.00 (*reference*) | | 1.00 (*reference*) | |
| **Disease-free Survival** | | | | | |
| MSI | 48 | 1.00 (0.68-1.45) | 0.99 | 1.18 (0.81-1.71) | 0.39 |
| MSS | 1202 | 1.00 (*reference*) | | 1.00 (*reference*) | |
| **Distant recurrence** | | | | | |
| MSI | 48 | 0.94 (0.54-1.63) | 0.94 | 0.98 (0.57-1.71) | 0.95 |
| MSS | 1202 | 1.00 (*reference*) | | 1.00 (*reference*) | |
| **Local recurrence** | | | | | |
| MSI | 48 | 1.52 (0.62-3.74) | 0.37 | 1.53 (0.60-3.86) | 0.40 |
| MSS | 1202 | 1.00 (*reference*) | | 1.00 (*reference*) | |

[a] *Adjusted for age, neoadjuvant treatment, adjuvant treatment, disease stage*

Currently, the addition of neoadjuvant treatment strategies to TME surgery is the standard of care for locally advanced rectal cancer. Therefore, the effect of MSI or MSS status on survival has been evaluated in a homogenous patient cohort of patient receiving neoadjuvant (chemo)radiotherapy and TME surgery selectively (*n*=746). The 5-year OS was 71% and 69% for patients with MSS and MSI tumors, respectively. Furthermore, after 5 years of follow-up 95% of the patients with MSS rectal cancer was local recurrence free compared with 83% of the patients with MSI rectal cancer tumors, however not significant.

## META-ANALYSIS OF PUBLISHED LITERATURE AND CURRENT STUDY

The search was performed in September 2017, resulting in 204 papers, after removal of duplications n=179. Title and abstract screening was performed and 151 paper were excluded (including 17 non-English papers, 2 papers about in vitro experiments, 2 papers in which no MSI was performed, 29 reviews and case reports, 34 cases not focused on rectal cancer). Based on full text review we included 14 original studies, that are summarized in table 3. The total number of MSI cases in these studies (including our own) is 317 (out of 5448 rectal cancer patients), with an overall prevalence of 5.8% (SE 2.3%). In figure 3 the prevalence of MSI cases per study is shown. In blue all studies that fall within the average (SE).

**Table 3.** Study characteristics

| Author (year) | Cohort[1] | Disease Stage | Cases total | Cases MSI | Type of outcome |
|---|---|---|---|---|---|
| own study | unselected | I-IV | 1250 | 48 | OS, DFS |
| Bae, 2013 [27] | unselected | I-IV | 168 | 5 | OS, DFS |
| Charara, 2004 [20] | unselected | unknown | 57 | 5 | PR (complete response) |
| Choi, 2007 [37] | unselected | unknown | 18 | 5 | PR (downstaging) |
| Colombino, 2002 [25] | unselected | I-III | 91 | 17 | none |
| Demes, 2013 [38] | unselected | I-IV | 25 | 2 | PR (response) |
| De Schoolmeester, 2008 [39] | unselected | I-III | 90 | 1 | none |
| Du, 2012 [28] | unselected | II-III | 316 | 25 | DFS, PR (downstaging) |
| Hong, 2011 [29] | unselected | I-IV | 465 | 20 | OS, DFS |
| Meng, 2007 [30] | unselected | II-III | 128 | 12 | OS |
| Phipps, 2013 [3] | familiar | unknown | 1111 | 37 | OS |
| Samowitz, 2009 [26] | unselected | I-IV | 979 | 22 | OS |
| Seppala, 2015[40] | unselected | I-IV | 197 | 6 | none |
| Yang, 2015[24] | unselected | II | 460 | 97 | DFS |
| Yoon, 2016[31] | unselected | II-III | 93 | 15 | DFS |

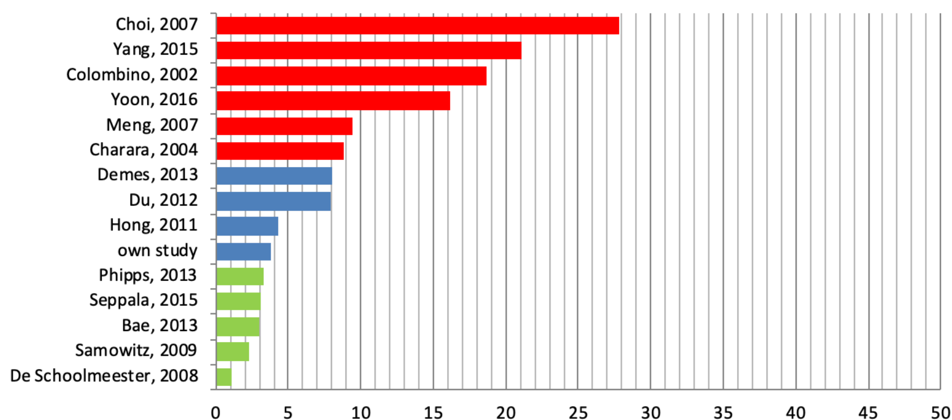
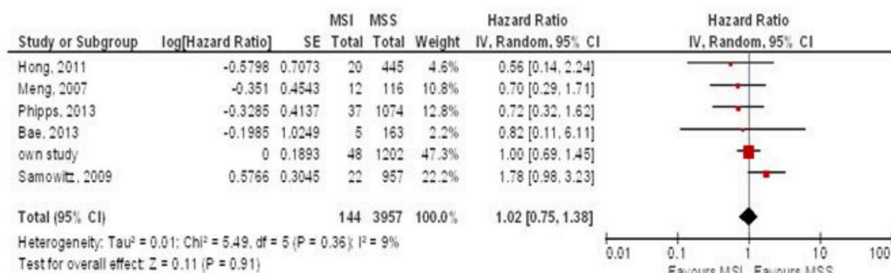
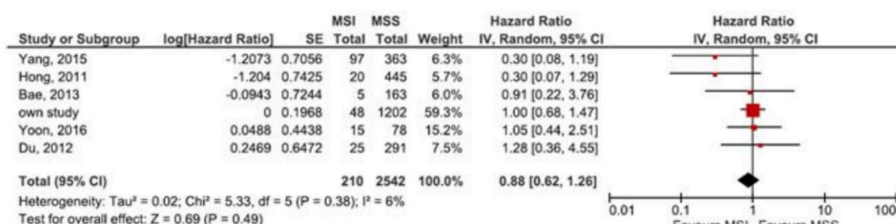



**Figure 3.** The prevalence of MSI cases per study. In blue all studies that fall within the average of 5.8% (±SE 2.3%).

Both for overall survival (figure 4A) as for disease free survival (figure 4B) there was no impact of MSI status on prognosis (HR 1.02, 95%CI 0.75-1.38 and HR 1.09, 95% CI 0.79-1.50, respectively). There was no heterogeneity between the studies. Response of neoadjuvant therapy was determined on very small numbers of MSI patients (n = 38),

with profound heterogeneity ($I^2$ = 68%) and different methods of response determination (see table 3). The overall risk ratio was 0.9 (95%CI 0.36-2.24), demonstrating no effect of MSI status on response to neoadjuvant therapy.



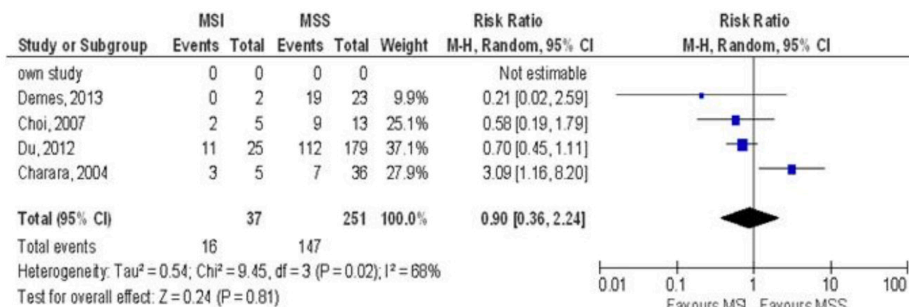




**Figure 4. The impact of MSI on outcome.** A. Univariate overall survival B. Univariate disease-free survival. C. Pathological response.

HR: Hazard ratio, CI: confidence interval, RR: relative risk.

# DISCUSSION

Routine screening for MSI in patients with newly diagnosed CRC has been supported by the ASCO and ESMO [10-12]. Due to the relative low incidence of MSI in rectal cancer, limited evidence regarding the prognostic and predictive value of MSI existed. We studied the prognostic value of microsatellite stability status in the largest rectal cancer cohort, currently available. Furthermore, the data was collected in a prospective manner because all patients were included in two randomized controlled trials [17,23]. In this study, MSI was present in 3.8% of the rectal cancer patients, which is in line with the literature. We observed no effect of MSI status on overall survival, disease-free survival, distant recurrence and local recurrence in patients stratified by microsatellite status. This is in line with the data obtained in our meta-analysis.

Particularly in early colon cancer, MSI is associated with a prognostic advantage [4-7]. For rectal cancer, only one of the included studies, reported a significant favorable survival (DFS) in patients with MSI rectal cancer compared with MSS [24]. An additional study, suggested a similar effect, however, the data provided in this study did not allow inclusion in our meta-analysis. Moreover, the population in this study showed a particularly high incidence of MSI in rectal cancer (19%), which might be caused by the high inbreeding rate and relative genetic homogeneity of the Sardinian population [25]. In contrast, *Samowitz et al*. reported a significant unfavorable prognosis in a subset of patients with MSI rectal cancers [26]. The majority of the studies did not observed a significant difference between MSI and MSS in rectal cancer, similar to our own study [3,27-31]. It is unclear why there is a difference between colon and rectal cancer in this respect. Differences in treatment strategies (including neoadjuvant treatment and superb surgical techniques) might be responsible for this.

Currently, neoadjuvant chemoradiation is the standard of care for locally advanced rectal cancer, of which 5-FU is used as the standard chemotherapy agent. In general, MSI predicts a poor response to 5-FU-based chemotherapy in colon cancer [9]. Response to neoadjuvant chemoradiation varies among patients, a significant pathological downstaging has been observed in approximately 20% of all cases [32,33]. The presence of MSI did not predict response, however, the number of cases in the literature is low. Interestingly, in a series of Lynch syndrome patients (n = 62), a pCR of 27.6%, following neoadjuvant 5-FU based chemoradiation was reported, which is higher than the common reported complete response rates [34]. From a molecular perspective, an intact DNA repair mechanism to induce apoptosis after 5-FU incorporation is required. For that reason it has been hypothesized that, colon cancer cells with a deficient mismatch repair mechanism showed resistance to treatment with 5-FU based chemotherapy [35,36].

On the other hand, 5-FU based chemotherapy could act as a strong radio-sensitizing agent in patients with MSI rectal cancer resulting in a pCR, as observed by *de Rosa et al.* [34].

Our study used a prospective randomized design in a large patient cohort, where we have performed state of the art analysis of MSI. However, we acknowledge that the performed study has some limitations. Due to the rarity of MSI in rectal cancer the sample size for analysis in subgroups is small. Due to continuous innovations in rectal cancer treatment it is difficult to study outcomes in small subgroups with similar treatment with sufficient power. By combining our analysis with all currently available evidence in the literature, we showed that MSI in rectal cancer is a rare event, without any impact on patients outcome.

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9

# 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.

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# Appendices

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# ALGEMENE DISCUSSIE

Gedurende de laatste twee decennia is de behandeling van patiënten met colorectale tumoren aanzienlijk verbeterd. Met name door de introductie van de totale mesorecale excisie (TME), gecombineerd met preoperatieve (chemo)radiatie voor patiënten met een rectumtumor, is het aantal lokale recidieven drastisch teruggebracht [1]. Voor maligniteiten gelokaliseerd in het colon (stadium III en hoog-risico stadium II tumoren) is na de introductie van adjuvante chemotherapie met 5-fluoruracil capecitabine en oxaliplatin de mortaliteit gedaald [2-4]. Tot voor kort werden zowel patiënten met een colontumor als met een rectumtumor behandeld met adjuvante chemotherapie. Echter staat adjuvante behandeling middels chemotherapie in patiënten met een rectumtumor ter discussie. In een veel geciteerde Japanse studie werd dit positieve effect van adjuvante chemotherapie beschreven. Noemenswaardig is het feit dat de patiënten in deze Japanse studie niet werden behandeld met preoperatieve (chemo) radiotherapie gevolgd door gestandaardiseerde TME chirurgie [5]. In 2012 verscheen een Cochrane review, waarin opnieuw een positief effect van adjuvante chemotherapie werd beschreven. Opvallend is dat in geen van de 21 geïncludeerde studies chirurgie middels het TME principe werd toegepast. Daarbij werd in slechts twee studies (chemo) radiotherapie toegepast [6] . In tegenstelling tot de geïncludeerde studies in de Cochrane review is in Nederland preoperatieve (chemo)radiatie gevolgd door TME chirurgie de standaard. Zoals eerder beschreven door Breugom *et al.* en later in een meta-analyse, opgenomen in dit proefschrift (hoofdstuk 2), kan in patiënten met stadium II-III rectumtumoren geen overlevingswinst worden aangetoond bij toepassing van adjuvante chemotherapie na behandeling met preoperatieve (chemo)radiatie en TME chirurgie [7-9]. Met name op basis van de resultaten uit hoofdstuk 2 kan gesteld worden dat er geen plaats is voor de behandeling met adjuvante chemotherapie voor patiënten met een rectumtumor. In de huidige richtlijnen is het pathologische stadium van de tumor leidend voor de toe te passen behandelstrategie [10]. Ondanks continue verbetering van het pathologisch classificatiesysteem (TNM classificatie) verschilt de klinische uitkomst tussen patiënten met dezelfde pathologisch geclassificeerde colorectale tumor [11]. Mede daarom zou het huidige stadiëringssysteem aangevuld moeten worden met biomarkers, om zodoende meer individuele behandelingen toe te kunnen passen en overbehandeling te voorkomen. In dit proefschrift worden naast het effect van adjuvante chemotherapie verschillende biomarkers bestudeerd.

Om de leesbaarheid van dit proefschrift te vergroten is het verdeeld in drie delen. In deel I wordt de uitvoering van een meta-analyse beschreven, waarin het effect van adjuvante chemotherapie, na preoperatieve (chemo)radiatie en TME chirurgie, in patiënten met stadium II-III rectumtumor werd geëvalueerd. In hoofdstuk 3 werd in het bijzonder

gekeken naar het effect van adjuvante chemotherapie in patiënten met rectumtumoren gelokaliseerd >10cm van de anus. In hoofdstuk 4 werden pathologische kenmerken van rectumtumoren bestudeerd, om zodoende patiënten te kunnen selecteren welke wel baat zouden kunnen hebben van adjuvante chemotherapie. Deel II van dit proefschrift beschrijft de expressie van eiwitten op colorectale tumorcellen, betrokken bij het ‘ontwijken’ van het immuunsysteem. De expressie van het klassieke HLA klasse I molecuul en het niet-klassieke HLA-G molecuul werden uitgebreid bestudeerd [12-15]. In deel III zijn kenmerken van colorectale tumoren bestudeerd op het niveau van genetica en epigenetica. In hoofdstuk 8 werd gebruik gemaakt van de LINE-1 methylatiestatus. De methylatiestatus van LINE-1, een repeterende sequentie in het DNA, kan gezien worden als surrogaat marker voor de methylatiestatus van het gehele genoom. Hypomethylatie van het genoom zou in maligniteiten geassocieerd kunnen zijn met een slechtere overleving. In hoofdstuk 9 werd de welbekende biomarker microsatelliet instabiliteit (MSI) bestudeerd. Deze, reeds in de kliniek gebruikte biomarker, voorspelt een goede prognose in vroeg stadium colontumoren. Ondanks het gebruik in de kliniek is deze biomarker tot op heden niet goed bestudeerd in een specifieke groep van patiënten met een rectumtumor. Daarom werd in hoofdstuk 8 het voorkomen van MSI positieve tumoren en de relatie tot overleving in deze patiëntengroep bestudeerd.

**DEEL I**

**ADJUVANTE CHEMOTHERAPIE VOOR HET LOKAAL GEVORDERDE RECTUMTUMOR**

De behandelstrategie van rectumtumoren wordt bepaald in een multidisciplinair team van medisch specialisten. De hoeksteen van de behandeling van niet gemetastaseerde rectumtumoren is chirurgisch ingrijpen, met name door het uitvoeren van de zogenoemde TME chirurgie. Door de invoering van TME chirurgie is de overleving van patiënten met een rectumtumor drastisch verbeterd [1]. Ondanks deze verbetering ontwikkelt ongeveer 30% van de patiënten metastasen op afstand [16-18]. Adjuvante chemotherapie zou in theorie de metastaserende cellen kunnen elimineren om derhalve metastasering te voorkomen. Echter, het te verwachten positieve effect van adjuvante chemotherapie voor de behandeling van patiënten met rectumtumoren werd geëxtrapoleerd vanuit het bewezen effect van adjuvante chemotherapie in patiënten met tumoren gelokaliseerd in het colon. Daarbij is het positieve effect van adjuvante chemotherapie na behandeling met preoperatieve (chemo)radiatie gevolgd door TME chirurgie in patiënten met rectumtumoren tot op heden niet aangetoond in gerandomiseerde studies [7-9]. Ondanks het feit dat het effect van adjuvante chemotherapie niet is bewezen in studieverband, worden patiënten ermee behandeld. Om robuust bewijs te leveren werd in hoofdstuk 2 van dit proefschrift een meta-analyse met individuele patiëntgegevens uitgevoerd, waarin adjuvante chemotherapie werd vergeleken met observatie na behandeling met preoperatieve (chemo)radiatie en

TME chirurgie in patiënten met een stadium II-III rectumtumor. Na een literatuurstudie werden vier gerandomiseerde fase III studies geïncludeerd. Deze studies vergeleken adjuvante chemotherapie met observatie in patiënten met niet gemetastaseerde rectumtumoren, welke behandeld waren met preoperatieve (chemo)radiatie en TME chirurgie. Het totaal aantal geïncludeerde patiënten in de vier studies was 2195, waarvan 1196 patiënten voldeden aan de inclusiecriteria. Samenvattend; op basis van hoofdstuk 2 kan worden geconcludeerd dat patiënten met een stadium II of III rectumtumor, behandeld met preoperatieve (chemo)radiatie en TME, chirurgie geen baat hebben van behandeling met adjuvante chemotherapie. Echter liet een subgroep analyse een opvallende bevinding zien; patiënten met rectumtumoren, gelokaliseerd tussen de 10-15cm vanaf de anus, hebben mogelijk wel overlevingswinst van behandeling met adjuvante chemotherapie. Nader onderzoek voor deze subgroep van patiënten is essentieel. Daarom werd in hoofdstuk 3 van dit proefschrift specifiek onderzoek gedaan naar patiënten met rectumtumoren gelokaliseerd tussen de 10 en 15cm vanaf de anus, geïncludeerd in de PROCTOR-SCRIPT studie. De PROCTOR-SCRIPT studie is een multicenter gerandomiseerde fase III trial, waarin werd gerandomiseerd tussen observatie of adjuvante chemotherapie bij patiënten met stadium (y)pTNM II-III rectumtumoren, behandeld met preoperatieve (chemo)radiatie en TME chirurgie. Overeenkomstig met de resultaten uit de meta-analyse werd een significant gunstig effect gezien van adjuvante chemotherapie ten opzichte van observatie op de ziektevrije overleving in patiënten met rectumtumoren tussen de 10 en 15cm vanaf de anus, in vergelijking met patiënten met rectumtumoren tussen de 0 en 10cm vanaf de anus (alle patiënten werden behandeld met preoperatieve (chemo)radiatie en TME chirurgie). Op basis van deze resultaten zou bediscussieerd kunnen worden of hoge rectumtumoren als colontumoren beschouwd moeten worden, aangezien patiënten met colontumoren wel baat hebben bij adjuvante chemotherapie.

In het kader van gepersonaliseerde zorg, zijn niet alleen macroscopische tumorkarakteristieken, maar ook microscopische tumorkarakteristieken van invloed. Zoals lymfatische invasie, perineurale invasie, veneuze invasie en tumor budding. Deze pathologische markers werden al eerder geassocieerd met een ongunstig ziektebeloop [19-23]. Bovendien zouden deze pathologische markers kunnen bijdragen aan een meer gepersonaliseerde behandelingsstrategie, zoals het gebruik van adjuvante chemotherapie [23-25]. In hoofdstuk 4 van dit proefschrift werd bevestigd dat de eerder genoemde stadiumonafhankelijke pathologische markers geassocieerd waren met een ongunstige uitkomst in een cohort met patiënten met een rectumtumor. Daarbij werd ook geconcludeerd dat overleving sterk geassocieerd was met de aanwezigheid van twee of meer van de genoemde pathologische markers. Een verklaring voor deze bevinding zou kunnen zijn dat patiënten met aanwezigheid van $\geq 2$ pathologische

markers meer "toegang" hebben tot de verschillende routes van metastaseringen. Tumorcellen kunnen zodoende langs de hematogene, lymfogene of langs betrokken zenuwen metastaseren. Een misschien nog wel belangrijkere bevinding in hoofdstuk 4 is dat deze pathologische markers geen predictieve rol spelen in patiënten met rectumtumoren. Anders gezegd, ondanks de aanwezigheid van deze ongunstige pathologische markers heeft behandeling met adjuvante chemotherapie in deze patiëntengroep geen positief effect. Naast de uitgevoerde studies in dit proefschrift is in de literatuur steeds meer bewijs voor een belangrijkere rol voor preoperatieve behandeling van rectumtumoren vergeleken met adjuvante behandeling [17, 26]. Daarbij wordt preoperatieve behandeling beter verdragen dan adjuvante behandelstrategieën. Op dit moment is chemoradiatie gevolgd door TME chirurgie de meest gebruikte therapie voor patiënten met rectumtumoren. Maar de optimale dosering van de chemoradiotherapie en timing van de daaropvolgende operatie zijn nog niet geoptimaliseerd [27]. In een recente studie van Erlandsson *et al.*, waarin drie preoperatieve radiotherapieregimes werden vergeleken, werden significante verschillen gezien ten aanzien van het aantal lokale recidieven, metastasen op afstand en algehele overleving [28]. Ook werden interessante resultaten beschreven ten aanzien van postoperatieve complicaties. In de groep patiënten behandeld met radiotherapie, waarbij de operatie werd uitgesteld tot 4-6 weken na de laatste behandeling, werden significant minder postoperatieve complicaties waargenomen. Wel werd 7% van de geïncludeerde patiënten in deze groep opgenomen met radiatietoxiciteit.

Samenvattend werd in deel I van dit proefschrift geen significant effect gezien van de behandeling met adjuvante chemotherapie na preoperatieve (chemo)radiatie en TME chirurgie in patiënten met stadium II-III rectumtumoren. Daarbij werd een prognostisch ongunstig effect waargenomen van pathologische markers (lymfatische invasie, perineurale invasie, veneuze invasie en tumor budding), maar kon geen predictieve waarde worden ontleed aan deze markers in relatie tot adjuvante chemotherapie. Verder is de huidige definitie van *het* rectum verschillend tussen landen; bijvoorbeeld gemeten in centimeters vanaf de anus of gedefinieerd ten aanzien van de peritoneale omslagplooi. Gebaseerd op de bevindingen uit hoofdstuk 2 en 3 rijst de vraag of rectumtumoren gelokaliseerd >10cm vanaf de anus als colontumoren moeten worden beschouwd, gezien het vergelijkbare prognostisch gunstige effect van adjuvante chemotherapie.

## DEEL II
## INTERACTIE TUSSEN HET IMMUUNSYSTEEM EN COLORECTALE TUMOREN, HLA-G EXPRESSIE

Zoals beschreven door Hanahan and Weinberg verkrijgen cellen in het proces van maligne transformatie de zes welbekende biologische kenmerken [29]. In 2011 werden hier twee nieuwe kenmerken aan toegevoegd, waarvan het omzeilen van het immuunsysteem door maligne cellen in dit deel van het proefschrift werd onderzocht [30]. De huidige hypothese is dat tumorcellen worden herkend door het immuunsysteem en zodoende worden geëlimineerd. Door dit systeem te omzeilen heeft een tumorcel de mogelijkheid om te metastaseren. Het niet-klassieke humaan leukocytenantigeen (HLA) klasse I molecuul HLA-G zou hierbij een rol kunnen spelen. In niet pathologische condities komt HLA-G tot expressie in immuun bevoorrechte weefsels zoals de placenta. *De novo* expressie van het HLA-G eiwit is beschreven in verschillende typen kanker, waaronder ook colorectale tumoren. HLA-G zou kunnen bijdragen aan het omzeilen van het immuunsysteem door het remmen van NK cellen en T-cel gemedieerde cellyse [15]. Daarom zou HLA-G geassocieerd kunnen zijn met een ongunstige prognose. Een tweede manier om het immuunsysteem te omzeilen is om HLA klasse 1 in mindere mate tot expressie te brengen op de tumorcel. Door middel van HLA klasse 1 eiwitten presenteren tumorcellen tumor-geassocieerde-antigenen op het celoppervlak. Deze antigenen worden door cytotoxische t-cellen herkend en vervolgens worden deze cellen gedegradeerd [9]. Deze vernietiging door cytotoxische T-cellen zou dus voorkomen kunnen worden door HLA-klasse 1 te downreguleren. Eerdere studies hebben laten zien dat downregulatie van HLA klasse 1 inderdaad geassocieerd is met een ongunstige overleving [31-33]. Bovendien werd bij colorectale tumoren met HLA klasse 1 downregulatie en HLA-G expressie een nog ongunstiger prognose gezien [31-33]. Deze bevindingen ondersteunen de hypothese van een verminderde opsporing van maligne cellen door het immuunsysteem, wanneer HLA klasse I moleculen minder tot expressie komen en HLA-G tot expressie komt. Om meer inzicht te verkrijgen in het immunologische profiel van metastaserende cellen werd in hoofdstuk 5 de expressie van HLA klasse 1 en HLA-G middels immuunhistochemie (IHC) gevisualiseerd en vergeleken in zowel de primaire colorectale tumor als de bijbehorende levermetastase. In tegenstelling tot de hypothese werd gevonden dat het overgrote deel van de cellen in de metastasen HLA klasse 1 tot expressie brachten. Daarbij werd geen associatie gezien tussen de primaire tumoren en bijbehorende levermetastasen, positief bevonden voor HLA-G eiwit expressie. Op basis van deze bevindingen kon geconcludeerd worden dat HLA klasse 1 downregulatie en *de novo* expressie van HLA-G zouden kunnen bijdragen aan het omzeilen van het immuunsysteem, maar zeker niet absoluut noodzakelijk voor de formatie van metastasen.

Ondanks het feit dat IHC in het algemeen een zeer aanvaarde laboratoriumtechniek is, blijft de detectie van HLA-G middels IHC controversieel. Bijvoorbeeld, het commercieel verkrijgbare en veelvuldig gebruikte moleculair antilichaam (mAb) 4H84 staat bekend om zijn kruisreactie met het ß2-microglubulin (ß2m) van HLA klasse 1 eiwitten op geactiveerde leukocyten [34]. Dit zou kunnen leiden tot vals positieve erkenning van het HLA-G eiwit in tumoren welke worden geïnfiltreerd met leukocyten, zoals vaak wordt gezien bij colorectale tumoren. Om deze reden wordt in de literatuur gesuggereerd om altijd meerdere mAbs te gebruiken om het HLA-G eiwit te visualiseren [34, 35]. Het is daarom van belang om te noemen dat de meerderheid van de uitgevoerde studies waarin HLA-G expressie wordt geëvalueerd alleen gebruik maken van mAb 4H84. In hoofdstuk 5 werd gebruik gemaakt van drie verschillende mAbs (4H84, MEM-G/1 en MEM-G/2); alle drie de mAbs zijn gericht tegen het HLA-G eiwit. Een zeer onverwachte en opvallende bevinding in dit hoofdstuk is het verschil in aankleuringspatroon tussen deze drie verschillende mAbs. Hiermee werd in hoofdstuk 5 het bestaan van niet-specifieke binding van de verschillende mAbs gericht tegen HLA-G aangetoond. Omdat IHC alleen niet afdoende lijkt te zijn werden aanvullende biochemische onderzoeken uitgevoerd om de aanwezigheid van het HLA-G molecuul in colorectale tumoren nader te onderzoeken. In hoofdstuk 6 werden 21 recent vervaardigde cellijnen met een zeer laag aantal passages gebruikt voor verder onderzoek naar HLA-G. In deze cellijnen werd gekeken naar de DNA methylatiestatus van het *HLA-G* gen en de aanwezigheid van HLA-G coderend mRNA. Daarbij werd HLA-G membraanexpressie onderzocht middels flowcytometrie en IHC. Voor IHC werd gebruik gemaakt van drie verschillende mAbs (4H84, MEM-G/1 en MEM-G/2). Bovendien werden niet alleen de cellijnen, maar ook de bijbehorende tumoren waarvan de cellijnen waren afgeleid, middels IHC onderzocht. Samenvattend werd er opvallend genoeg geen correlatie gevonden tussen de hoeveelheid DNA methylatie van het *HLA-G* gen en de aanwezigheid van HLA-G coderend mRNA. In de uitgevoerde polymerase chain reaction (PCR) werd in 6 van de 21 cellijnen een zwak signaal gevonden. Na sequensen van het PCR product werd in vier van deze cellijnen een homologie gezien met HLA-G3, een van de HLA-G isovormen. In overeenstemming met de resultaten gevonden met PCR werd geen HLA-G1 expressie waargenomen bij analyse middels flow-cytometrie. Ook werd geen associatie gezien tussen met IHC aankleurende cellijnen en de bijbehorende primaire tumor. Evenals in hoofdstuk 5 werd in hoofdstuk 6 opvallende discrepantie gezien in aankleuring met IHC tussen de drie anti-HLA-G mAbs. Ondanks het feit dat HLA-G als een veelbelovend immune-checkpoint molecuul wordt gezien, kan worden geconcludeerd dat de huidige detectietechnieken nog niet selectief genoeg zijn om alle aspecten van het HLA-G eiwit in colorectale tumoren te ontrafelen. In hoofdstuk 7 werd verder uitgezocht hoe de discrepantie tussen de aankleuring met IHC verklaard zou kunnen worden. De eerder gebruikte cellijnen werden nu nogmaals gebruikt voor

analyse middels de western-blot techniek [36]. Aanvullend werd de HLA-G expressie bepaald op vers ingevroren colorectale tumorweefsels. De resultaten verkregen met western-blot analyse werden vergeleken met de klassieke IHC methode. Ook in dit hoofdstuk werd gebruikt gemaakt van verschillende mAbs gericht tegen alle HLA-G isovormen, zoals 4H84 en MEM-G/1, en mAb 5A6G7 gericht tegen de oplosbare HLA-G moleculen. In hoofdstuk 7 werd gezien dat de resultaten verkregen met western-blot niet vergelijkbaar zijn met de resultaten verkregen middels IHC op hetzelfde weefsel. Daarbij werden met de western-blot methode additionele eiwit bandjes waargenomen, welke in molecuul gewicht niet corresponderen met HLA-G. Dit fenomeen werd waargenomen in experimenten uitgevoerd met de drie eerder genoemde mAbs. Gebaseerd op hoofdstuk 5, 6 en 7 kan worden geconcludeerd dat de rol van HLA-G als immuun modulator in colorectale tumoren prematuur is. Tot het moment dat het technisch mogelijk is om HLA-G expressie op betrouwbare wijze te detecteren in biologische samples in plaats van in getransfecteerde cellijnen of in langdurig gekweekte cellijnen, zullen conclusies met grote voorzichtigheid getrokken moeten worden en zijn therapeutische implicaties voor het HLA-G eiwit nog niet op zijn plaats.

### DEEL III
### GENETISCHE EN EPIGENETISCHE BIOMARKERS IN COLORECTALE TUMOREN

Hypomethylatie van het genoom wordt geassocieerd met een nadelige prognose in patiënten met colorectale tumoren [37-41]. Om genoomwijde hypomethylatie te meten wordt gebruik gemaakt van LINE-1 (Long Interspersed Element-1) als surrogaat marker [42]. LINE-1 repeterende elementen zijn aanwezig op nagenoeg alle chromosomen en beslaan ongeveer 17% van het gehele humane genoom [43]. In colorectale tumoren zou hypomethylatie geassocieerd zijn met een slechtere prognose [40]. Daarbij werd gezien dat hypomethylatie toeneemt in meer voortgeschreden tumorstadia [37, 41]. Eerder werd een prognostische waarde gezien in vroeg stadium rectumtumoren. Echter, tot op heden ontbrak gedegen onderzoek in een patiënten cohort bestaande uit specifiek vroeg stadium colontumoren. In hoofdstuk 8 werd de prognostische waarde van LINE-1 methylatiestatus onderzocht in patiënten met stadium II colon tumoren (n=164). Patiënten met een lage LINE-1 methylatiestatus (hypomethylatie) hadden een significant slechtere overleving dan patiënten met een hogere methylatiestatus. Dit effect werd in sterkere mate waargenomen bij patiënten boven de 65 jaar. Deze bevindingen steunen de hypothese dat LINE-1 methylatiestatus gebruikt zou kunnen worden als prognostische marker. Opvallend genoeg werd het waargenomen verschil in overleving niet teruggezien in ziektevrije overleving. Daarom kan op basis van deze studie geen prognostische waarde aan LINE-1 hypomethylatie worden toegeschreven. Ook in de huidige literatuur wordt frequent geen relatie gevonden

tussen overleving en methylatiestatus. Wel wordt hypomethylatie vaak waargenomen en vooral in meer gevorderde ziektestadia [38, 41, 44]. Het lijkt dus zo te zijn dat algeheel verlies van DNA methylatie een vroege gebeurtenis is in de vorming van colonkanker, in plaats van een bijdragende factor aan progressie van de ziekte. Deze hypothese wordt ondersteund door de resultaten van Pavicic *et al*. [45]. Zij evalueerden de LINE-1 methylatiestatus in normale epitheelcellen van patiënten met hereditary nonpolyposis colorectal cancer (HNPCC), familiare colorectale tumoren en sporadische colorectale tumoren. De laagste methylatiestatus werd gevonden in de normale epitheelcellen van patiënten met familiare colorectale tumoren. Dit suggereert dat de mucosa door de hypomethylatie is gepredisponeerd voor tumor ontwikkeling. Verder zijn interessante studies gepubliceerd over de hypothese dat de methylatiestatus van het DNA in het colon een leeftijdsgebonden aspect heeft [46, 47]. Dit suggereert dat het verlies van DNA methylatie toeneemt met de leeftijd en derhalve bijdraagt aan de stijgende incidentie van colorectale tumoren met de leeftijd [48, 49]. Door het toenemende verlies van DNA methylatie met de leeftijd kan de leeftijd van een persoon geschat worden op basis van het verlies aan methylatie. Beschreven werd dat een discrepantie tussen kalenderleeftijd en geschatte leeftijd op basis van DNA methylatie (bijvoorbeeld; de voorspelde leeftijd op basis van methylatiestatus is hoger dan de huidige kalenderleeftijd) is geassocieerd met een hogere mortaliteit [48, 49]. Op basis van deze literatuur zou globale hypomethylatie kunnen functioneren als een "hit" in de "multi-hit hypothese" van Knodson. Hierom zou globale hypomethylatie meer beschouwd kunnen worden als een "driver" van carcinogenese, in plaats van een prognostische factor zoals beschreven werd gezien in hoofdstuk 8.

Een ander zeer bekende genetische marker die bijdraagt aan colorectale kankerformatie is MSI (microsatelliet instabiliteit). MSI is geassocieerd met een prognostisch voordeel in vroeg stadium colorectale tumoren [50-52]. Bovendien werd in patiënten met MSI colontumor resistentie tegen 5-FU gebaseerde chemotherapie gezien [51-54]. Daarom werd zeer recent standaard screening op MSI aangeraden door de richtlijnen van de American Society of Clinical Oncology (ASCO) en de European Society for Medical Oncology (ESMO) [55, 56]. In tegenstelling tot colontumoren zijn de implicaties van MSI in rectumtumoren tot op heden niet opgehelderd. Dit is echter zeer relevant om toe te kunnen werken naar gepersonaliseerde behandelstrategieën gedreven door biomarkers. Door de relatief lage incidentie van MSI in rectumtumoren bestaat er gelimiteerd bewijs aangaande de prognostische en predictieve waarde van MSI in rectumtumoren. Daarom werden in hoofdstuk 9 de prevalentie en de prognostische waarde van MSI bepaald in het tot nu toe grootste cohort van patiënten met rectumtumoren. Deze patiënten waren eerder geïncludeerd in twee prospectieve fase III studies (TME trial en PROCTOR-SCRIPT trial). In lijn met de reeds bestaande literatuur werd MSI gevonden

in 3.8% van de patiënten met rectumtumoren. Zowel op de overleving als het ontstaan van afstandsmetastasen of lokale recidieven werd geen verschil gevonden tussen patiënten met MSI of MSS rectumtumoren. Zoals beschreven in hoofdstuk 9, is de literatuur rondom de prognostische waarde van MSI in rectumtumoren conflicterend. Bijvoorbeeld Colombino *et al.* beschrijft een significant betere overleving voor patiënten met MSI rectumtumoren [57] . Daarentegen wordt door bijvoorbeeld Samowitz *et al.* een significant slechtere overleving beschreven voor patiënten met MSI rectumtumoren [58]. De laatste decennia is de behandeling van rectumtumoren sterk veranderd. Door de verandering in behandelstrategie is het lastig om de bestaande literatuur te vergelijken. Hierdoor bestaat een gebrek aan bewijs voor patiënten met MSI of MSS rectumtumoren behandeld met de huidige conventionele therapie. Samenvattend zijn MSI rectumtumoren een aparte subklasse van colorectale tumoren. Gezien de lage incidentie van MSI in rectumtumoren zal het echter een uitdaging zijn om de invloed van MSI op prognose geheel te kunnen evalueren.

## TOEKOMSTPERSPECTIEF

Het baseren van behandelstrategieën op enkel de huidige TNM classificatie heeft niet de potentie om de meest optimale en meer geïndividualiseerde patiëntenzorg te leveren. De conventionele stadiëring moet daarom aangevuld worden met biomarkers om zodoende meer ‘personalized medicine’ toe te kunnen passen. Ondanks intensief onderzoek naar genetische, epigenetische, moleculaire en pathologische markers schiet de klinische toepassing van biomarkers in de dagelijkse praktijk tekort. Gedurende het proces van tumorontwikkeling verwerven tumorcelen meerdere eigenschappen. Daarom zouden meerdere biomarkers gecombineerd moeten worden voor prognostische en predictieve doeleinden. Het gebruik van een enkele prognostische of predictieve biomarker is zeker niet afdoende. Daarbij zou biomarkeronderzoek waardevoller worden wanneer het uitgevoerd kan worden op biopten genomen voor de start van de behandeling, in het bijzonder voor rectumtumoren. Informatie verkregen uit biopten zou de timing en toepassing van preoperatieve behandeling kunnen beïnvloeden. Door de huidige behandeling van rectumtumoren met preoperatieve (chemo)radiatie en TME chirurgie is het aantal lokale recidieven flink gedaald. Een groot effect op algehele overleving werd echter nog niet waargenomen. Zoals beschreven in hoofdstuk 2 laat de toevoeging van adjuvante chemotherapie na behandeling met preoperatieve (chemo)radiatie en TME chirurgie geen verbetering zien op de overleving. Een alternatief voor het elimineren van micrometastasen in de behandeling van rectumtumoren zou systemische therapie voorafgaand aan de operatie kunnen zijn. Deze verschuiving naar meer intensievere

preoperatieve behandeling is op dit moment gaande en de resultaten hiervan zullen worden beschreven in de RAPIDO trial. Preoperatieve therapie wordt beter verdragen dan postoperatieve therapie. Bovendien wordt bij meer intensievere preoperatieve behandeling van rectumtumoren een complete pathologische respons waargenomen in ongeveer 30% van de patiënten. Daaruit rijst de vraag of een grote operatie vermeden zou kunnen worden in patiënten met een rectumtumor. De zogenoemde 'watch-and-wait' benadering geïntroduceerd door Harb-Gama en recent beschreven door van der Valk *et al*. laat zien dat in een strikt geselecteerde patiënten met een complete pathologische respons de watch-and-wait benadering sterk te overwegen valt [59, 60]. Een andere veelbelovende toekomstige toepassing van biomarkers in de behandeling van colorectale tumoren is het peroperatief visualiseren van tumorweefsel. Door middel van tumor-specifiek contrastmiddel, zoals Surgimab (een monoclonaal antilichaam met fluorescent label gericht tegen het carcinoembryonic antigen (CEA)) kan tumorweefsel in real-time worden gevisualiseerd om zodoende radicale resectie te bewerkstelligen [61]. De ultieme uitdaging voor de komende jaren is om kennis rondom het voorspellen van therapierespons in patiënten met een rectumtumor te vergaren door klinische, pathologische en moleculaire markers te combineren. Daarvoor zal intensieve samenwerking nodig zijn tussen de verschillende medisch specialisten om zodoende de meest effectieve therapie toe te passen, toegespitst op de individuele patiënt in plaats van een patiëntengroep.

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# Appendices

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# LIST OF PUBLICATIONS

**Swets, M**., A. Wouters, D. Krijgsman, R. L. P. van Vlierberghe, A. Boot, J. D. van Eendenburg, T. van Wezel, H. Gelderblom, C. J. H. van de Velde, P. J. van den Elsen and P. J. K. Kuppen (2018). “HLA-G protein expression in colorectal cancer evaluated by immunohistochemistry and western blot analysis: Its expression characteristics remain enigmatic.” Clin Immunol 194: 80-86.

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# CURRICULUM VITAE

Marloes Swets was born on March 20th 1987 in Voorburg, the Netherlands. After graduating from the Interconfessioneel Makeblijde College (IMC) in Rijswijk in 2005 she studied biomedical sciences at the Leiden University for one year, before she started Medical school at the Leiden University Medical Centre in 2006. After completing her preclinical training she started her clinical rotations in 2010. During her studies she did an internship at the department of dermatology, at the Biomedicum, Helsinki, Finland. In 2012 she performed her graduation research at laboratory of Gastroenterology and Hepatology at the Leiden University Medical Centre, under supervision of prof. dr. D.W. Hommes and prof. dr. ir. H.W. Verspaget. After obtaining her medical degree in January 2013, she started working at the department of internal medicine of the IJsselland Hospital, Capelle aan den IJssel, under supervision of dr. H.E. van der Wiel, internist. At the IJsselland Hospital she was a member of the tissue and organ donation committee. In May 2014, she started with her PhD training at the department of Surgical Oncology and Medical Oncology at the Leiden University Medical Centre under supervision professor C.J.H. van de Velde, professor A.J. Gelderblom and dr. P.J.K. Kuppen. The results of her studies are presented in this thesis and have been published in several international journals. In May 2017, after finalizing her graduate program, Marloes started her residency training in Gastroenterology and Hepatology at the Leiden University Medical Centre under supervision of dr. A.M.J. Langers, the Haaglanden Medical Centre (HMC) in the Hague under supervision of dr. A.H. Bootsma and currently at the HagaZiekenhuis in the Hague under supervision of  dr. A. Bhalla.

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## DANKWOORD

Dit proefschrift is mede tot stand gekomen dankzij het werk en de steun van velen, waarvan ik er een paar in het bijzonder wil noemen.

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