Biomarkers in colorectal cancer

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Chapter 9

No effect of microsatellite status on outcome of rectal cancer

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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.

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).

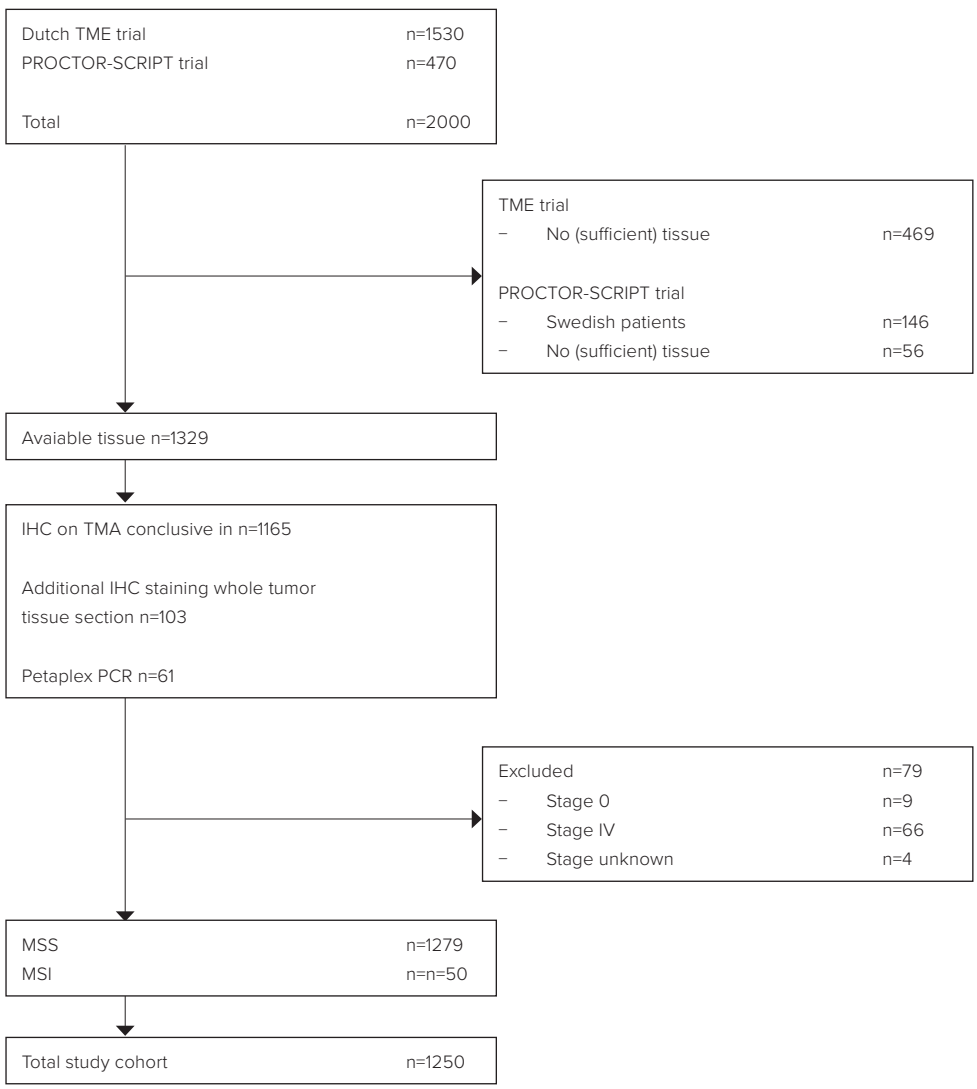


Figure 1. Patient selection.

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 χ^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).

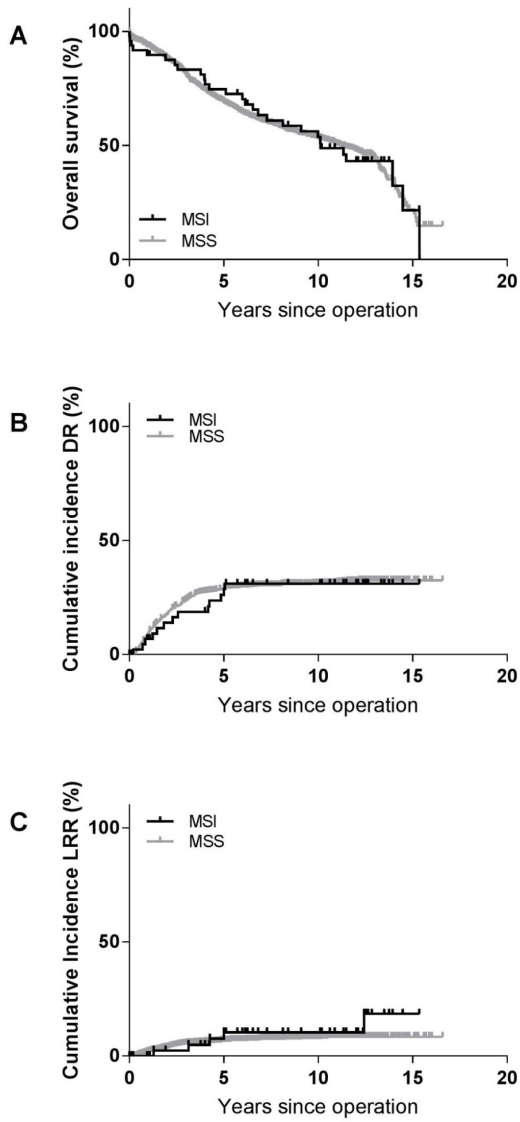


Figure 2. Kaplan-Meier analysis for overall survival (A), time to distant recurrence (B) en time to local recurrence (C) in 1250 rectal cancer patients according to MSS or MSI (n=48) status.

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

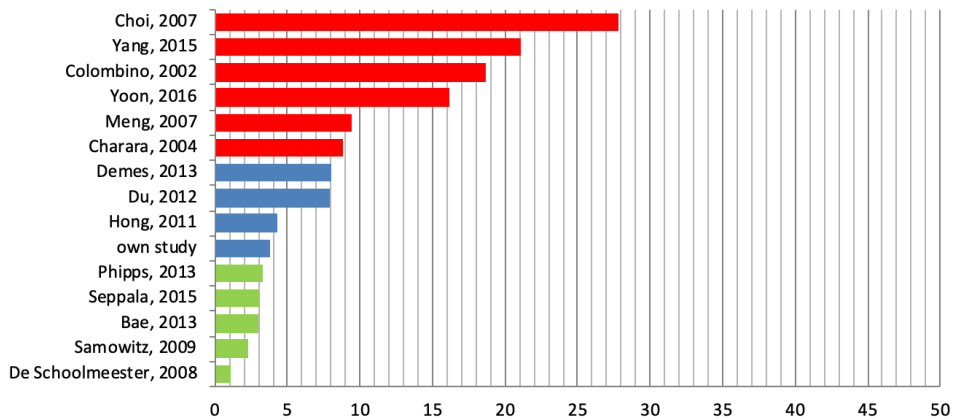
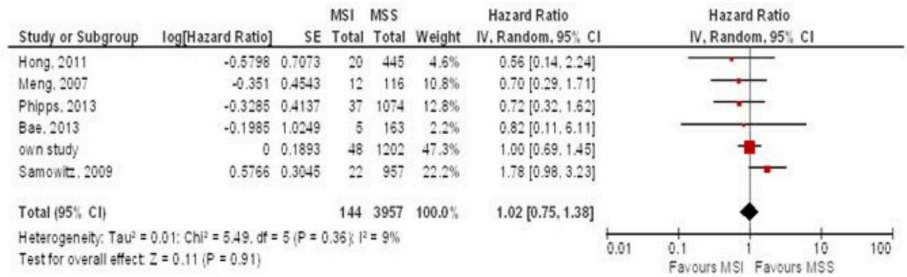
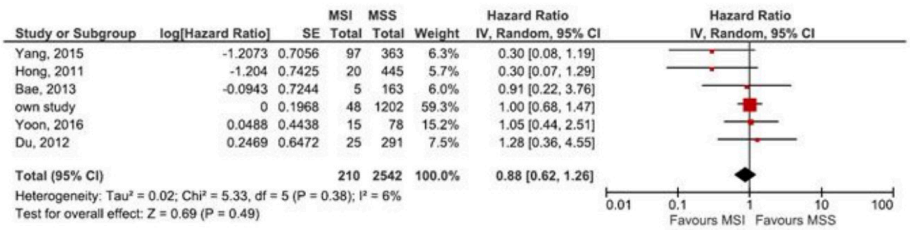


Figure 3. The prevalence of MSI cases per study. In blue all studies that fall within the average of 5.8% (±SE 2.3%).

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.





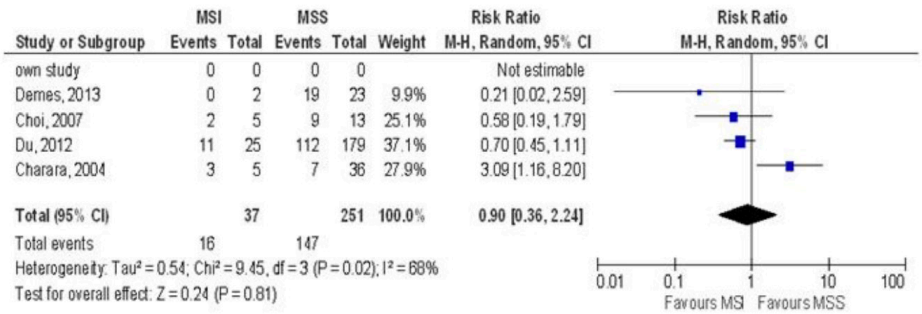


Figure 4. The impact of MSI on outcome. A. Univariate overall survival B. Univariate disease-free survival. C. Pathological response.

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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