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ORIGINAL ARTICLE

Mismatch repair protein expression is an independent prognostic factor in sporadic colorectal cancer

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Abstract

Background. Mismatch repair (MMR) status has been reported as a prognostic and predictive factor in sporadic colorectal cancer (CRC). The purpose of this study was to determine the prognostic and predictive value of MMR protein expression in the adjuvant setting. *Patients and methods.* The MMR status in the primary tumor was retrospectively assessed on paraffin-embedded formalin-fixed samples from 1 006 patients with sporadic CRC (488 stage II and 518 stage III) using immunohistochemical analysis (IHC) of MLH1 and MSH2 expression. The patients were included in adjuvant Nordic trials between 1991 and 1996 randomly assigned to surgery alone or surgery plus adjuvant 5-fluorouracil (5-FU)-based chemotherapy. Data was censored at 120 months after surgery. *Results.* One hundred fifty-seven patients (15.6%) showed a loss of MMR protein expression (139 MLH1 negative, 15 MSH2 negative and 3 MLH1 and MSH2 negative) and were classified as MMR protein negative. A normal MMR protein expression was found in 849 patients who were defined as MMR protein positive. MMR protein expression was a significant prognostic marker in the entire study group with a better overall survival (OS) among patients with MMR protein negative tumors compared to patients with MMR protein positive tumors ($p=0.01$). In a multivariate analysis the MMR protein expression was significantly associated with OS, (HR 0.70 [95% CI, 0.40 to 0.99]; $p=0.01$). The MMR status did not predict survival benefit from adjuvant 5-FU-based chemotherapy. *Conclusion.* This study reveals that IHC of MLH1 and MSH2 expression can yield important prognostic information but is not a predictive factor for adjuvant 5-FU-based chemotherapy in sporadic CRC.

Colorectal cancer (CRC) is the third most common malignancy in the Western world. Despite advances in treatment CRC is still a leading cause of cancer-related death [1]. Recent studies have revealed the potential use of molecular and biochemical markers in CRC to predict outcome and response to chemotherapy [2]. The mismatch repair (MMR) status has been reported as one promising marker.

Fearon and Vogelstein have described two major pathways of CRC development according to the form of genetic instability involved [3]. The CRC carcinogenesis is usually a result of mutations in the adenomatous polyposis coli (APC) gene which are characterized by chromosomal instability (CIN). However, 10–15% of all sporadic colorectal cancers arise via the microsatellite instability pathway (MSI). MSI is a manifestation of a defect in one of several DNA MMR genes, including MLH1 and MSH2. The leading cause of hereditary nonpolyposis colorectal carcinoma (HNPCC) is inherited defects in DNA MMR genes where MSI is present in more than 90% of the patients. MSI in sporadic CRC is most commonly a result of an epigenetic biallelic methylation of the promoter sequences of MLH1 [4]. Tumors arising from the two separate pathways CIN and MSI show distinct clinical and pathological features. Sporadic MSI tumors tend to be proximal and usually have a diploid phenotype whereas sporadic CIN tumors often have no site predilection and are aneuploid. Histologically sporadic MSI tumors exhibit poor differentiation, a mucinous cell type and peritumoral lymphocytic infiltration [5].

Several studies have shown that immunohistochemical (IHC) assessment of MLH1 and MSH2

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protein expression has a high degree of correlation with the MSI phenotype determined by PCR. The sensitivity to detect a MSI tumor with IHC is 80–95% and the specificity has reached 100% in most reports [6–10].

Multiple studies have demonstrated that sporadic CRC with MSI have a better prognosis than patients with CRC exhibiting microsatellite stability [11–15,20]. The relationship between MSI tumors and a better survival has also been confirmed in a systematic review [16].

The value of MSI to predict the response to chemotherapy is still controversial. The chemotherapeutic regimen for CRC is based on thymidine analogues such as 5-FU, which causes DNA damage. It is known that DNA MMR deficient cell lines are resistant to DNA damage and consequently also to 5-FU [17]. Retrospective studies have demonstrated an improved survival in patients with MMR competent tumors after receiving 5-FU based chemotherapy [18–21]. The first prospective trial also indicates a survival benefit among colorectal cancer patients with MMR competent tumors treated with 5-FU [22,23].

The aim of this study was to exclusively evaluate the role of MMR status as a prognostic factor as well as a predictive factor of benefit from adjuvant chemotherapy with 5-FU in stage II and III CRC patients included in a randomized trial.

Patients and methods

The Nordic colorectal group enrolled 2 224 patients with CRC (stage II and III) in adjuvant clinical trials from 1991 to 1996 [24]. Only patients up to the age of 75 years and with no history of other malignancies within the last five years were included. Tissue from the primary tumors of 1 042 Swedish patients was available for analysis. Thirty-six (3.4%) of the 1 042 patients were excluded from the study because the quality of the immunostaining was unsatisfactory and this study includes the remaining 1 006 patients. The patients included in our study are representative of the patients who participated in the Nordic trials in terms of baseline characteristics. The patients were randomly assigned to either surgery alone or surgery plus adjuvant 5-FU based chemotherapy according to the Moertel schedule with levamisole, the Mayo regimen with or without levamisole or the Nordic schedule with or without levamisole. The adjuvant chemotherapy was started within 10 weeks after surgery. The tumor samples were obtained from 59 different hospitals in Sweden. Patients' characteristics are listed in Table I. The median age of the patients was 66 years (range 24–75 years). Two hundred and twenty-five patients of the 492 patients (46%) treated with chemotherapy received more than 90% of the planned dose of 5-FU included in the given chemotherapeutic regimen. Data was censored at 120 months after surgery and parameters of clinical outcome were obtained from the centers of Epidemiological Oncology.

Laboratory methods

Two formalin-fixed paraffin-embedded, tissue samples from different parts of each resected primary CRC tumor were used for this study. For the IHC of MLH1 and MSH2 all tumors were stained using the mouse immunoglobulin G monoclonal antibodies clone G168-15 (BD PharMingen, San Diego, CA, USA) at 1:100 dilution, and clone FE11 (Oncogene Research Products, Boston, MA, USA) at 1:100 dilution. Four micrometer sections are mounted on SuperFrost Plus glass slides dried at room temperature and then baked for one hour in 60°C, deparaffinized in xylene, rehydrated in graded ethanol, and washed in distilled water. For antigen retrieval, the slides were boiled in the microwave for 20 min in 1 mM EDTA (pH 9.0) and afterwards cooled down for 20 min in the same buffer at room temperature.

The slides were incubated with the primary antibody overnight in 4°C and thereafter incubated with an amplification system with a labelled polymer/HRP, EnVision™, (DakoCytomation, Denmark) for 30 min. To visualize the sites of bound peroxidase 0.05% 3, 3′-diaminobenzidine tetrahydrochloride (DAB) was used before counterstaining with a modified Harris hematoxylin.

Scoring

All tumor samples were examined under a light microscope and scored by two independent observers blinded to clinical and pathological data. A case was considered negative for expression of a given protein if the tumor cells displayed a complete absence of nuclear staining with the respective monoclonal antibody. Intact nuclear staining of normal tissue (non-neoplastic stromal cells and lymphocytes) adjacent to the tumor was used as internal positive control. The MSH2 antibody staining showed a nuclear staining that was easy to interpret. However, the MLH1 antibody yielded a few cases with a patchy staining. The agreement in evaluation between the two observers was > 90%. In case of disagreement the final score was determined by consensus after re-examination.

Statistics

Statistical differences in distribution of basal patients' characteristics between groups were calculated using

Table I. Patients' characteristics with respect to mismatch repair protein expression.

Characteristic	No. of Patients (n = 1006)	No. of Patients MMR protein neg (n = 157)	% MMR protein neg	No. of Patients MMR protein pos (n = 849)	% MMR protein pos	P-value
Age						NS
<66	502	88	17.5	414	82.5	
>66	504	69	13.7	435	86.3	
Sex						0.05
Male	559	76	13.6	483	86.4	
Female	447	81	18.1	366	81.9	
Tumour site						<0.001
Proximal colon*	390	113	29.0	277	71.0	
Distal colon	328	29	8.8	299	91.2	
Rectum	288	15	5.2	273	94.8	
Stage						NS
II	488	84	17.2	404	82.8	
III	518	73	14.1	445	85.9	
Grade of differentiation						0.002
Poor	207	49	23.7	158	76.3	
Moderate	691	96	13.9	595	86.1	
Well	68	8	11.8	60	88.2	
Missing data	40	4	10.0	36	90.0	
Adjuvant chemotherapy						NS
With	491	82	16.7	409	83.3	
Without	515	75	14.6	440	85.4	
Numbers of analyzed LN**						
0–11	677	96	14.2	581	85.8	p=0.02
>11	87	21	24.1	66	75.9	
Missing data	242	40	16.5	202	83.5	
Local recurrence						
With	91	9	9.9	82	90.1	NS
Without	915	148	16.2	767	83.8	
Distant metastases						
With	264	30	11.4	234	88.6	p=0.03
Without	742	127	17.1	615	82.9	

Abbreviations: MMR, mismatch repair; NS, not significant (p>0.05). P-values for the comparison between patients without MMR protein expression and patients with MMR protein expression were analyzed using the $\chi 2$ test. *, To splenic flexure. **, Data available for 764 patients.

the χ^2 test. The Gehan-Wilcoxon univariate test was used to examine the possible correlation between overall survival (OS), disease-free survival (DFS) and MMR-protein expression. Multivariate analyses were performed using Cox proportional hazards regression models. Survival curves were estimated using the Kaplan-Meier method. OS was defined as the time from surgery to death of any cause whereas DFS was defined as the time from surgery to the first relapse or death of any cause.

Results

Patient characteristics

This study included 1 006 patients with stage II and III CRC. Seven hundred and eighteen patients (71%) had a primary tumor located in the colon and 288 (29%) in the rectum (Table I). In the entire study cohort 305/1 006 (30%) of the patients developed local tumor recurrence or distant metastases. There was a significant difference between stage II and III CRC in the number of patients who were diagnosed with local tumor recurrence or distant metastases, stage II 93/488 patients (19%) versus stage III 212/518 patients (41%) p=0.02. In the entire study population the median OS was 84 months. The 5-year survival rate in the entire study group was 64.1%. The median OS for patients with stage II disease was 91 months and for patients with stage III disease 65 months. The median OS in the subgroup of patients treated with surgery alone was 79 months. In patients treated with adjuvant chemotherapy the median OS was 86 months. During 120 months follow-up 463 patients (46%) died of whom 376 patients (81%) died with disseminated CRC.

MLH1 and MSH2 expression and patient characteristics

Among the 1 006 patients who were analysed with respect to MLH1 and MSH2, 157 patients (15.6%) showed a complete loss of protein expression. One

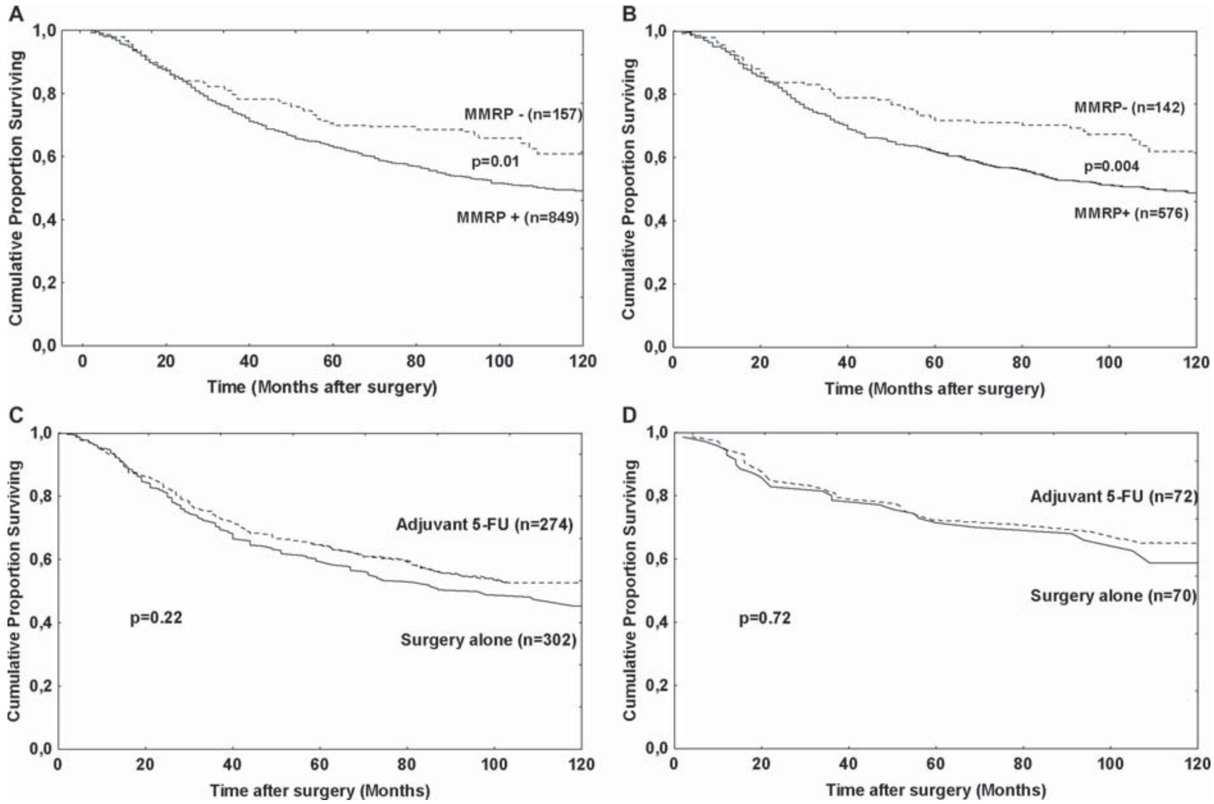

Figure 1. (A) Overall survival for the entire study group of 1 006 colorectal cancer (CRC) patients with respect to mismatch repair protein expression (MMRP). (B) Overall survival for the subgroup of 718 colon cancer patients with respect to mismatch repair protein expression (MMRP). (C) Overall survival for the subgroup of patients with MMR positive colon cancer (n=576) according to treatment status. (D) Overall survival for the subgroup patients with MMR negative colon cancer (n=142) according to treatment status.

hundred and thirty nine patients (13.8%) had MLH1 negative tumors and 15 (1.5%) demonstrated a total loss of the MSH2 protein. Only three patients lacked protein expression of both MLH1 and MSH2. The relationship between clinic pathologic features and MLH1 and MSH2 expression is listed in Table I. A loss of MLH1/MSH2 protein expression was associated with gender, where MMR-protein negative carcinomas occurred more frequently in women ($p=0.05$). In addition MMR-protein negative tumors were primarily localized in the colon 142/157 (90%, $p< 0.001$) and mainly proximal to the splenic flexure 113/157 (72%, $p<0.001$). The loss of MLH1 or MSH2 expression was also characterized by poor differentiation ($p=0.002$). With respect to age and stage of disease no significant difference was found between patients according to their MMR-status. The development of distant metastases (n=264) was more common in patients whose primary tumor was MMR protein positive 234/264 (89%) compared with whose tumors where MMR protein negative 30/264 (11%, $p=0.03$). Patients with MMR protein positive tumors were also more likely to be diagnosed with a local recurrence 82/91 (90%) compared to patients with MMR negative tumors 9/91 (10%) but this difference was not significant.

MLH1 and MSH2 expression and clinical outcome

MMR-protein expression was a prognostic marker for the entire study population with a significant better median OS of 89 months in patients with MMR-protein negative tumors, compared to 82 months in patients with MMR protein positive tumors ($p=0.01$) (Figure 1A). The prognostic value of MMR protein expression was also significant in the subgroup of patients with colon cancer (n=718, $p=0.004$), Figure 1B. The survival advantage was especially pronounced in patients with MMR-protein negative tumors localized in the right or transverse colon ($p=0.0001$). In the subgroup of 488 CRC patients with stage II disease the MMR-status showed a trend towards a prognostic value ($p=0.07$) with a median OS of 95 months for MMR protein negative tumors compared to 91 months for patients with MMR protein positive tumors. However, in stage II colon cancer (n=356) the MMR-status was a significant prognostic marker ($p=0.05$). Among the 518 patients with stage III CRC there was a 22 month difference in median

OS (85 months versus 63 months) between patients with MMR protein negative tumors (n=73) and patients with MMR protein positive tumors (n= 445) but this difference was not significant. Neither did MMR-protein expression have a prognostic value in stage III colon cancer (n=362).

In a multivariate analysis the MMR protein expression was significantly associated with OS, (HR, 0.70 [95% CI, 0.40 to 0.99]; p=0.01) (Table II). A multivariate analysis was also performed to evaluate other prognostic factors in the study group such as gender (p=0.004), age (p<0.001), grade of differentiation (p=0.001), stage of disease (p<0.001) and numbers of analyzed lymph nodes (p=0.02).

MLH1 and MSH2 expression as predictor of benefit from adjuvant chemotherapy

In the group of 491 CRC patients who received adjuvant chemotherapy the MMR protein expression did not have a predictive value for response to 5-FU with respect to OS. Neither did MMR protein expression predict response to 5-FU in the subgroup of patients with colon cancer (Figure 1C, D).

Discussion

The current staging and risk stratification methods in CRC are insufficient. The use of prognostic and predictive markers would enable the clinicians to tailor the optimal treatment for each individual patient maximizing the therapeutic effect and minimizing toxicity.

It is hard to compare the results between studies addressing the prognostic and predictive role of MSI in CRC as various methods (IHC and PCR polymerase chain reaction) have been used to determine MSI in different tumor locations (colon and rectum) and treatment settings (adjuvant and palliative). Furthermore, most of these studies have not included patients from randomized clinical and the numbers of patients in each study is usually small. The majority of the previous studies have used PCR to identify MSI. However, IHC determination of MLH1 and MSH2 is becoming a widely used technique that has made it possible to easily at a low cost define the MMR status in paraffin-embedded formalin fixed tissue and can be carried out even if only a small amount of tumor tissue is available [6–10]. Other advantages with IHC are the abilities to evaluate histological features of the tumors and tumor heterogeneity. On the other hand, IHC will always be semi-quantitative and therefore difficult to standardize.

In this present study with 1 006 stage II and III patients included in a randomized trial we retrospectively showed that IHC of MLH1 and MSH2 can be used to give prognostic information in sporadic CRC. We found a significant better OS in patients with MMR protein negative carcinomas compared to patients with MMR protein positive tumors in univariate as well as multivariate analysis of the entire study population (Table II). In accordance with the multivariate analysis described in two other studies we also found age, sex, stage of disease and grade of differentiation to be independent prognostic markers [13,20]. In contrast to the previous studies reporting a survival benefit for patients with tumors exhibiting MSI we also included the numbers of analyzed lymph nodes in the multivariate analysis [11–15,20]. In our study cohort more than 11 examined lymph nodes were a significant prognostic marker. It has been previously described that a higher number of lymph nodes harvested is associated with a better survival in CRC [25]. According to most guidelines, the recommended number of lymph nodes examined to ensure a proper staging is 12 nodes or more [26].

The importance of the results from this current study exclusively analyzing patients with stage II and III disease in a large sample size collected under clinical trial conditions has to be underlined. In the systematic review that confirmed an association between MSI in CRC and a better prognosis only

Table II. Univariate & Multivariate analysis using Cox Proportional Hazards Model to identify significant variables and generate HR for death.

	Univariate analysis			Multivariate analysis		
	HR for death	95% CI	P-value	HR for death	95% CI	P-value
Age (<66 vs. >66)	1.33	1.14–1.51	0.002	1.43	1.24–1.61	<0.001
Sex (female vs. male)	0.77	0.58–0.96	0.006	0.76	0.57–0.95	0.004
Tumor site (colon vs. rectum)	0.98	0.79–1.17	NS	0.86	0.66–1.07	NS
Stage (III vs. II)	2.21	2.02–2.41	<0.001	2.12	1.92–2.31	<0.001
Grade of differentiation (well/moderate vs. poor)	0.81	0.71–0.93	<0.001	0.72	0.53–0.92	0.001
Treatment (chemotherapy vs. no chemotherapy)	0.88	0.70–1.06	NS	0.90	0.72–1.08	NS
Number of analyzed LN (≥11 vs. 0–11)	0.60	0.21–0.98	0.008	0.62	0.23–1.00	0.02
MMR protein status (negative vs. positive)	0.68	0.40–0.96	0.01	0.70	0.40–0.99	0.01

Abbreviations: MMR, mismatch repair; MMR positive, MLH1 or MSH2 positive; MMR negative, MLH1 negative or MSH2 negative; HR, hazard ratio; 95% CI, 95% confidence interval; NS, not significant (p>0.05).

13/32 (41%) of the contributing studies described patients with stage II and III CRC [16].

A non-randomized study including 718 patients with CRC showed a better clinical outcome in patients with a negative MMR-protein expression in their tumors [13]. Among patients with MMR protein negative tumors a significant better clinical outcome was also found in stage II and III disease. The survival advantage was especially evident in patients with MMR-protein negative Stage II tumors localized in the proximal colon. However, the number of patients included in this subgroup analysis is not stated in the report. In an abstract Tejpar et al. describe analysis of the MSI-status in 1 327 colon cancer patients from the adjuvant PETACC-3 trial treated with 5-FU or 5-FU and irinotecan. The data from this large study cohort also demonstrate that microsatellite instability is a strong prognostic factor for both stage II and III colon cancer [15]. In our study the survival advantage was also significantly enhanced in patients with MMR-protein negative tumors localized in the proximal colon, (n=113). We could also see a significant survival benefit in the subgroup of patients with stage II colon cancer (n=356).

The distinct clinical features in sporadic MSI CRC may reflect the different genetic pathways of carcinogenesis and support the hypothesis of another mechanism of cancer development in the proximal compared to the distal colon [5]. In 30–35% of the carcinomas of the proximal colon there is an abnormal MMR protein expression compared to only 10–15% among all colon cancers. If the proximal and distal parts of the colon represent distinct entities this might have potential implications in the therapeutic approach [27].

In vitro studies have shown that CRC cells with MSI are less responsive to 5-FU [17]. Consistent with these preclinical results Ribic et al. reported in a randomized retrospective study a survival advantage in 5-FU treated CRC patients with MSI-L and MSS cancers but not in patients with MSI-H tumors [18]. Two other non-randomized retrospective studies including 204 and 1263 CRC patients respectively also found a benefit of 5-FU in patients without MSI [19,20]. Deficient mismatch-repair as a predictive marker for lack of benefit from 5-FU based chemotherapy in stage II and III colon cancer has also been shown in a pooled molecular reanalysis of randomized chemotherapy trials (n=341) [21]. In contrast with these findings early clinical trials indicated that 5-FU was beneficial for CRC patients with MSI tumors [28,29]. However, without a control group of patients who did not receive any chemotherapy it is hard to evaluate whether the improved survival was due to the MMR-status in the tumors or the chemotherapy in itself. The first prospective study of the use of adjuvant 5-FU chemotherapy based on the MMR status was published by Jover et al. 2006 [22]. The 754 patients in this study were a nested cohort from the large EPICOLON trial investigating the prevalence of Lynch syndrome in Spain. Consistent with previous retrospective data a better overall survival was found among CRC patients with MMR proficient tumors treated with 5-FU adjuvant chemotherapy. This data has recently been confirmed on the same cohort of patients prospectively followed during five years [23]. In our retrospective analysis in a randomized cohort of CRC patients MMR protein expression did not predict clinical outcome of adjuvant 5-FU with respect to OS. The 1 006 patients in our report represent a group of Swedish patients of a total cohort of 2 224 stage II and III CRC patients enrolled in Nordic adjuvant trials randomly assigned to either surgery alone or surgery plus adjuvant chemotherapy. Among the 2 224 patients in the Nordic trials a non-significant absolute difference of 7% favouring chemotherapy was found in colon cancer stage III [24]. However, among the Norwegian cohort of patients (n=425) the subgroup of colon cancer patients with stage III disease (n=105) exhibited a significant survival difference in favor for adjuvant 5-FU-based chemotherapy according to the Moertel scheme [30]. According to the general consensus there is a significant survival advantage in colon cancer stage III treated with adjuvant chemotherapy [31]. The absence of a significant survival benefit from adjuvant chemotherapy in the entire Nordic study cohort has to be taken into account while analyzing the predictive utility of MMR protein expression and adjuvant 5-FU in this study. The effect of adjuvant chemotherapy is also a result of the accumulated dose of chemotherapy administered to each patient. In our study 225/491 (46%) of the patients who were treated with adjuvant chemotherapy received more than 90% of the planned 5-FU dose. Furthermore, the adjuvant chemotherapy included five different 5-FU regimens, which might have had an impact on the results [24]. The time interval until start of adjuvant chemotherapy has also to be considered where a delay longer than eight weeks has been reported to be negative [32]. The median time to initiation of adjuvant treatment in the Nordic trials was seven weeks which was longer than in any of the previous reported randomized adjuvant trials showing a survival benefit of 5-FU [24].

Another retrospective study from 2007 with data from prior National Surgical Adjuvant Breast and Bowel Project (NSABP) Collaboration Studies does not support the use of MSI as a predictor of benefit of chemotherapy [33]. However, it is important to be aware of the high risk of selection bias in this retrospective study where patients treated with chemotherapy and the untreated controls were collected from separate clinical trials. A recently published study showed interestingly that loss of MMR function may predict improved outcome in stage III colon cancer patients treated adjuvantly with irinotecan and 5-FU compared with those patients only receiving 5-FU [34].

For many decades clinical management as well as research has defined CRC as a homogenous entity instead of a complex heterogeneous disease. In a review article the pathologist Jass describes five different subtypes of CRC based on the correlation of clinical, morphological and molecular features [5]. This approach where MSI as an important molecular feature is a corner-stone seems very tempting and could have an impact on future treatment strategies in CRC.

The need of prognostic and predictive markers in CRC is non-questionable. It is essential to use robust quality controlled assays to identify new markers and their interpretation has to be standardized. This current study has revealed the prognostic value of MMR protein expression in sporadic stage II and III CRC. Even if the MMR expression was unable to predict outcome of adjuvant chemotherapy in our study cohort the results from previous trials are very promising. However, before MMR status can be implemented as a prognostic and predictive marker in clinical practice its value also has to be proven in large high-powered prospective trials.

Watanabe et al. have described that the presence of MSI, together with retention of the 18q allele and the transforming growth-factor-beta 1 mutation, is associated with a higher 5-year survival rate in stage III colon cancer [35]. The benefit of these markers in stage II colon cancer patients has not been shown. This question is being addressed in the ongoing prospective Intergroup E5202 study where stage II patients are stratified based on the presence of MSI and loss of heterozygosity 18q allele. Low-risk patients are observed, whereas high-risk patients (MSS with 18qLOH and MSI-L with 18qLOH) are randomized to FOLFOX with or without bevacizumab [36]. Results from this study will hopefully contribute to a better understanding of the role of MMR status as a predictive factor.

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