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ORIGINAL ARTICLE

Clinical impact of microsatellite instability in colon cancer following adjuvant FOLFOX therapy

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Abstract
Purpose Colon cancer with DNA mismatch repair (MMR) defects reveals indistinguishable clinical and pathologic aspects, including better prognosis and reduced response to 5-fluorouracil (5-FU)-based chemotherapy. There has been no consensus for p53 as a prognostic marker in colorectal cancer. This study investigated the clinical implication of MSI-H/MMR-D and p53 expression in R0-resected colon cancer patients who received adjuvant oxaliplatin/5-FU/leucovorin (FOLFOX) therapy.
Experimental design We analyzed 135 patients, who had been treated by adjuvant chemotherapy containing 5-FU and oxaliplatin (FOLFOX) after curative resection (R0) for colon adenocarcinoma between May 2004 and November 2007. Tumor expression of the MMR proteins, MLH1 and MSH2, was detected by immunohistochemistry (IHC) in surgically resected tumor specimens. MSI was analyzed by polymerase chain reaction (PCR) amplification using fluorescent dye-labeled primers specific for microsatellite loci. Tumors with MMR defects were defined as those demonstrating loss of MMR protein expression (MMR-D) and/or microsatellite instability high (MSI-H) genotype. Expression patterns of p53 were determined in a semi-quantitative manner by light microscopy.
Results There were 13 (9.6%) patients with stage II, 108 (80%) with stage III, and 14 (10.4%) with stage IV. Fourteen patients with stage IV (10.3%) had metastases to liver only, all of whom underwent complete metastasectomy for liver metastases. In total, 134 tumor specimens were genotyped, 115 specimens were tested by IHC and 113 cases had both genotyping and IHC results available for analysis. Genotyping results demonstrated that 12 (9.0%) cases were MSI-H and 122 (91.0%) were MSI-L/S. By IHC, 11 (9.6%) patients were MMR-D and 104 (90.4%) were MMR-I. The methods were in agreement in 108 patients (94.7%). We assessed 114 patients for p53 expression by immunostaining. MMR status was not significantly associated with DFS ($P = 0.56$) or OS ($P = 0.61$) in patients with colon cancer ($n = 135$) receiving adjuvant FOLFOX. According to p53 status, there was also no significant difference for DFS ($P = 0.11$) and OS ($P = 0.94$). For patients with genotyping/IHC agreement ($n = 108$), there was no difference in DFS ($P = 0.57$) and OS ($P = 0.98$) between patients with MSI-H/MMR-D and MSI-L/S/MMR-I tumors.
Conclusion The MMR status or p53 positivity was not significantly associated with outcomes to FOLFOX as adjuvant chemotherapy in colon cancer patients with R0 resection. Adding oxaliplatin in adjuvant chemotherapy

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may overcome negative impact of 5-FU on colon cancers with MSI-H/MMR-D.

Keywords MMR · MSI · FOLFOX · Adjuvant chemotherapy

Abbreviations

MMR	DNA mismatch repair
MMR-D	MMR-deficient
MMR-I	MMR-intact
MSI	The microsatellite instability
MSI-H	High levels of MSI
MSI-L	MSI-low
MSS or MSI-S	MSI-stable

Introduction

Colorectal cancer develops either sporadically (85%) or as part of a hereditary cancer syndrome (less than 10%), or against a background of inflammatory bowel disease. It is believed that the adenoma-carcinoma sequence underlies the development of colorectal cancer in most patients, and two distinct pathways have been identified—the microsatellite instability (MSI) and the chromosomal instability (CIN) pathways [12, 13, 16, 21]. The microsatellite instability (MSI) pathway, which involves failure of the nucleotide mismatch recognition and repair system, is one form of genomic instability [1, 19]. Deficient mismatch repair occurs in approximately 10–15% of all sporadic colorectal cancer [33]. In most sporadic cases, MSI occur when the promoter region of the mismatch repair gene, MLH1, is silenced by CpG island hypermethylation, and immunohistochemistry (IHC) identification of MLH1 and MSH2, two MMR proteins most commonly lost in sporadic colon cancer, is a widely available clinical test to distinguish MMR-deficient (MMR-D) from mismatch repair intact (MMR-I) tumors [17, 18, 25]. High levels of MSI (MSI-H) colorectal cancers are more frequent in women and more commonly located in proximal to the splenic flexure [19, 20, 24, 36]. The MSI-H colorectal cancers are known to bear many features that are generally associated with poor prognosis, including deep tumor invasion and poor histologic differentiation. However, patients with MSI-H tumor revealed longer overall and cancer-specific survival than stage-matched patients with tumor exhibiting CIN [15, 30], which implicated that the pronounced genetic instability of tumor cells with MSI may increase susceptibility to apoptosis.

The loss of DNA mismatch repair (MMR) proteins that had DNA damage sensor function leads to the lack of appropriate signals for apoptosis induction [4, 14] and resistance to specific chemotherapy drugs [2, 10, 22]. In studies for adjuvant chemotherapy in stage II or III colon cancer, patients with MSI-H demonstrated no benefit with regimen containing fluorouracil (FU) unlike patients whose tumors demonstrate CIN [5, 23, 31]. Recently, Bertagnolli et al. [6] reported that equalization of outcome for MSI-H and non-MSI-H tumors treated by irinotecan (CPT-11) and 5-FU as adjuvant chemotherapy regimen was observed and thus, concluded that the addition of irinotecan overcame a negative impact of 5-FU on MSI-H tumors in adjuvant setting. In current clinical practice, 6 months of postoperative 5-FU, LV, and oxaliplatin (FOLFOX) have been widely used as standard adjuvant chemotherapy regimen [37]. However, the clinical impact of MMR status on treatment response to FOLFOX as adjuvant regimen has not been evaluated.

P53 tumor suppressor gene is involved in the regulation of apoptosis. P53 interferes with the expression of a number of proapoptotic genes at the transcriptional level. P53 expression in colorectal carcinomas was known to be high [8, 26]. About 50–75% of colorectal carcinomas reveal positive for the p53 protein. This genetic marker has drawn attention among researchers. A pooled analysis including survival data from 4,416 patients in 28 published studies demonstrated that neither p53 expression nor p53 mutations emerged as powerful prognostic factors in patients with the disease [28]. However, there has been no consensus for p53 as a prognostic marker in colorectal cancer.

We intended to investigate whether the tumor MMR status have the association with outcomes to FOLFOX as adjuvant chemotherapy in colon cancer patients with R0 resection. MMR status was based on genotyping (MSI-H vs. MSI-L/S) and IHC (MMR-D vs. MMR-I). Moreover, we evaluated the significance of p53 expression as a prognostic marker in colon cancer patients with R0 resection.

Patients and methods

Patients

Between May 2004 and November 2007, 135 patients had been treated by adjuvant FOLFOX chemotherapy after curative resection (R0) for colorectal adenocarcinoma at Samsung Medical Center, Seoul, Korea. Of 135 patients, 14 had stage IV disease with complete resected synchronous liver metastasis. All 135 patients had been assessed for the status of MMR protein or MSI genotype. The following clinical data were collected from medical records for each patient: surgical and pathologic reports, imaging,

treatment modalities, the status of MMR protein and MSI genotype, and expression of p53. Staging of disease was classified according to the sixth edition of guidelines of the American Joint Committee on Cancer (AJCC).

Treatment

After R0 resection, all patients received FOLFOX. Oxaliplatin (85 mg/m^2 over 2 h, day 1), followed by leucovorin (200 mg/m^2 over 2 h, day 1) and then followed by 5-FU (400 mg/m^2 bolus, day 1 and then 2,400 mg/m^2 over 48 h, day 1 and 2) intravenous infusion. Cycles were repeated every 2 weeks until 12 cycles.

Analysis of MSI

Primary tissue specimens were obtained during surgery. Laboratory analysis was conducted at Samsung Medical Center. MSI was analyzed by polymerase chain reaction (PCR) amplification using fluorescent dye-labeled primers for the Bethesda markers (BAT-26, BAT-25, D5S346, D2S123 and D17S250) specific for microsatellite loci, as recommended by the National Cancer Institute Workshop on MSI [9]. MSI was defined as a band shift in either of the two alleles or as the appearance of a differently sized band in the tumor sample. Tumors were classified by MSI-H if instability was found at $\geq$50% of the loci screened, MSI-low (MSI-L) if at least one but $\leq$50% of the loci showed instability, and microsatellite stable (MSS or MSI-S) if all loci were stable. Immunohistochemistry (IHC) detected the presence of MLH1 and MSH2 protein in resected tumor specimens. Two MMR proteins (MLH1 and MSH2) were lost most commonly in sporadic colon cancer. For each antibody, a known positive normal colonic mucosa served as positive control. Tumors known to lack MLH1 or MSH2 served as negative control. All cases were scored as positive (defined as $\geq$10% of tumor cells staining) or negative (<10% tumor cells staining). The loss of MMR protein (MLH1 and/or MSH2) expression was defined as MMR-deficient (MMR-D) distinguishable from mismatch repair intact (MMR-I). Tumors with MMR defects were those demonstrating loss of MMR protein expression (MMR-D) and/or microsatellite instability high (MSI-H) genotype.

Table 1 Characterization of tumor microsatellite status

Characteristics	Immunohistochemistry		Genotyping	
	No.	%	No.	%
No. of cases attempted	115		134	
Result				
MMR-D (MSI-H)	11	9.5	12	8.9
MMR-I (MSI-L/S)	104	90.5	122	91.1

Table 2 Comparison of analysis methods

Genotyping results	Immunohistochemical result	
	MMR-D	MMR-I
MSI-H	8	3
MSI-L/S	3	100

Immunohistochemistry and immunostaining

Formalin-fixed, paraffin embedded tissues including both tumors and nontumorous liver tissues were sectioned at 4 μm. Immunohistochemical studies were performed using the streptavidin–biotin complex method and a TechMate^{TM} 1000 automated staining system (DakoChemmate, Glostrup, Denmark). Primary monoclonal antibody against p53 (clone Bp53-12) was purchased from Zymed Laboratories Inc. (San Francisco, CA, USA) and used at 1:400 dilution. Deparaffinized sections were treated with 3% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase. Sections were processed in 0.05 mol citrate buffer (pH 6.0) and heated in a microwave oven for 10 min for antigen retrieval. Sections were then incubated with the primary antibody for 30 min at room temperature. Secondary antibody (Dako, REAL^{TM}, EnVision^{TM}) was purchased from Zymed Laboratories Inc. (San Francisco, CA, USA). DAB (3,3′-diaminobenzidine tetrahydrochloride) was used as a chromogen, and Meyer's hematoxylin counterstain was applied.

Expression patterns of p53 were determined in a semiquantitative manner by light microscopy. Immunoreactivity for p53 (nuclear staining) was categorized in accordance with the percentage of tumor cells stained as described previously: immunonegative, $\leq$5%; immunopositive, >5% staining. Brown nuclear stain was regarded as positive.

Statistical analysis

Primary statistical outcomes were overall survival and disease-free survival (OS/DFS) measured from the date of surgery; DFS and OS were estimated by Kaplan–Meier curves and curves were compared by means of the log-rank test. Relationships between tumor MMR status and p53 expression and clinicopathologic factors were studied using the χ^2 test. The proportional hazards model was used to make survival comparisons controlling for treatment and other clinicopathologic factors. Statistical significance was defined as a two sided $P < 0.05$.

Table 3 Clinicopathological characteristics

	$N = 135$	Genotyping ($N = 134$)			IHC ($N = 115$)			P53 ($N = 114$)		
		MSI-H $N = 12$ (%)	MSI-L/S $N = 122$ (%)	P	MMR-D $N = 11$ (%)	MMR-I $N = 104$ (%)	P	Positive $N = 89$ (%)	Negative $N = 25$ (%)	P
Sex										
Male	84	9 (75)	75 (61)	0.53	6 (55)	63 (61)	0.75	34 (38)	11 (44)	0.60
Female	51	3 (25)	47 (39)		5 (45)	41 (39)		55 (62)	14 (56)	
Age, years										
≤65	101	10 (83)	90 (74)	0.73	9 (82)	74 (71)	0.73	63 (71)	19 (76)	0.61
>65	34	2 (17)	32 (26)		2 (18)	30 (29)		26 (29)	6 (24)	
T category										
T1–2	8	1 (8)	7 (6)	0.54	0 (0)	7 (7)	1.00	4 (4)	3 (12)	0.17
T3–4	127	11 (92)	115 (94)		11 (100)	97 (93)		85 (96)	22 (88)	
N category										
N0–1	77	4 (33)	72 (59)	0.08	6 (55)	57 (55)	0.98	44 (49)	18 (72)	0.04
N2	58	8 (67)	50 (41)		5 (45)	47 (45)		45 (51)	7 (28)	
Differentiation										
G1–2	17	5 (42)	12 (10)	<0.01	4 (36)	10 (10)	0.03	14 (16)	0 (0)	0.04
G3–4	118	7 (58)	110 (90)		7 (64)	94 (90)		75 (84)	25 (100)	
Stage										
II	13	3 (25)	10 (8)	0.04	3 (27)	7 (7)	0.02	7 (8)	3 (12)	0.36
III	108	9 (75)	98 (80)		8 (73)	83 (80)		70 (79)	20 (80)	
IV	14	–	14 (12)		0 (0)	14 (13)		12 (13)	2 (8)	
Location										
Right	47	7 (58)	40 (33)	0.11	6(55)	32(31)	0.17	60(67)	16(64)	0.75
Left	88	5 (42)	82 (67)		5(45)	72(69)		29(33)	9(36)	

Results

Microsatellite stability status and p53 status

In total, 134 tumor specimens were genotyped, 115 specimens were tested by IHC and 113 cases had both genotyping and IHC results available for analysis. Genotyping results demonstrated that 12 (9.0%) cases were MSI-H and 122 (91.0%) were MSI-L/S. By IHC, 11 (9.6%) patients were MMR-D (MSI-H) and 104 (90.4%) were MMR-I (MSI-L/S) (Table 1). The results from genotyping and IHC were concordant in 108 patients (94.7%). We assessed 114 patients for p53 expression by immunostaining (Table 2). The median age of the patients was 56 years (range, 30–78), and the majority (62%) of patients was male (Table 3). There were 13 (9.6%) patients with stage II, 108 (80%) with stage III and 14 (10.4%) with stage IV. At the time of analysis, tumor relapse was documented in 28.9% (39/135) of patients. Fourteen patients with stage IV (10.3%) had metastases to liver only, all of whom underwent complete metastasectomy for liver metastases.

Correlation between tumor MMR status and clinicopathologic factors

There were no significant difference in sex, age, T stage, N stage, and primary tumor site according to tumor MMR status (MSI-H vs. MSI-L/S). Patients with MSI-H tumors had significantly more poorly/undifferentiated tumors ($P < 0.01$) and early staged disease (χ^2, $P = 0.04$). Similarly, there were no significant differences between MMR-D and MMR-I tumors by sex, age, T stage, N stage, and primary tumor site. Patients with MMR-D tumors had significantly more poorly/undifferentiated tumors (χ^2, $P = 0.03$) and early staged disease (χ^2, $P = 0.02$). All patients ($n = 14$) with liver metastases had tumors identified as both MSI-L/S by DNA analysis and MMR-I by IHC. Except patients with stage IV, there was no significant difference between MSI-H and MSI-L/S and between MMR-D and MMR-I by stage. According to the status of p53 expression, there were no significant difference between p53 positive and p53 negative tumors by sex, age, category stage, N stage, and location. Patients with p53 negative tumors had significantly more

Table 4 Univariate analysis of DFS and OS in relation to clinicopathological parameters, genotyping, IHC, MMR status, and p53

	$N = 135$	3-year DFS (%)	P	HR (95% CI)	3-year OS (%)	P	HR (95% CI)
Sex							
Male	84	63.3		1.56 (0.78–3.15)	84.3		2.39 (0.67–8.48)
Female	51	75.9	0.20		94.0	0.16	
Age, years							
≤65	101	69.6	0.55	0.81 (0.40–1.63)	90.8	0.12	0.45 (0.16–1.27)
>65	34	62.6%			79.6		
T category							
T1–2	8	83.3	0.32	0.38 (0.05–2.78)	100	0.35	0.05 (0.0–10.0)
T3–4	127	66.8			87.2		
N category							
N0–1	77	70.9	0.13	0.62 (0.33–1.15)	94.8	0.01	0.23 (0.07–0.74)
N2	58	63.8			78.8		
Differentiation							
G1–2	17	61.7		1.25 (0.52–2.98)	80.7		0.98 (0.22–4.33)
G3–4	118	68.9	0.61		88.7	0.97	
Stage							
II	13	55		0.33 (0.10–1.05)	92.3	<0.01	0.36 (0.07–1.88)
III	108	75.3	<0.01		90.5		
IV	14	28.6			62.9		
Location							
Right	47	72.4	0.58	1.207 (0.61–2.38)	84.7		0.63 (0.23–1.73)
Left	88	65.5			89.8	0.36	
Genotyping							
MSI-H	12	66.7		0.79 (0.28–2.22)	90.9	0.76	1.38 (0.18–10.49)
MSI-L/S	122	67.5	0.65		87.4		
IHC							
MMR-D	11	63.6		0.69 (0.24–1.97)	88.9	0.79	1.31 (0.17–9.98)
MMR-I	104	65.6	0.49		84.4		
MMR status							
MMR-D and/or MSI-H	15	66.7		0.76 (0.29–1.94)	92.3	0.61	1.68 (0.22–12.78)
MMR-I and MSI-L/S	120	67.7	0.56		87.2		
P53 status							
Positive	89	60.7		0.43 (0.15–1.23)	84.5	0.94	0.96 (0.27–3.42)
Negative	25	82.6	0.56		85.3		

poorly/undifferentiated tumors (χ^2, $P = 0.04$) and nodal status of NO-1 (χ^2, $P = 0.04$).

MMR, p53 status, and survival

For 135 patients, the median follow-up duration was 2.3 years (range 24.1–32.1 months). The clinicopathological variable significantly associated with DFS was stage 3-year DFS (%); (stage II vs. III vs. IV = 55% vs. 75.3% vs. 28.6%, $n = 135$, respectively) (Table 4.). According to grouping by genotyping, IHC, MMR status, and p53 status in all 135 patients, there was no significant difference in terms of DFS and OS (Table 4; Fig. 1). In multivariate analysis, tumor stage was the only independent factor for predicting DFS (HR: 0.25, 95% CI, 0.11–0.56, $P = 0.002$) and tumor stage and nodal status were two independent factors separately for predicting OS (HR: 0.19, 95% CI, 0.06–0.63, $P = 0.021$, HR: 0.13, 95% CI, 0.03–0.61, $P = 0.010$, respectively). For patients with genotyping/IHC agreement ($n = 108$), there was no difference in DFS ($P = 0.57$) and OS ($P = 0.98$) between patients with MSI-H/MMR-D and MSI-L/S/MMR-I tumors (Fig. 2).

For stage II and III ($n = 121$), no significant difference was found by genotyping, IHC, MMR status, and p53

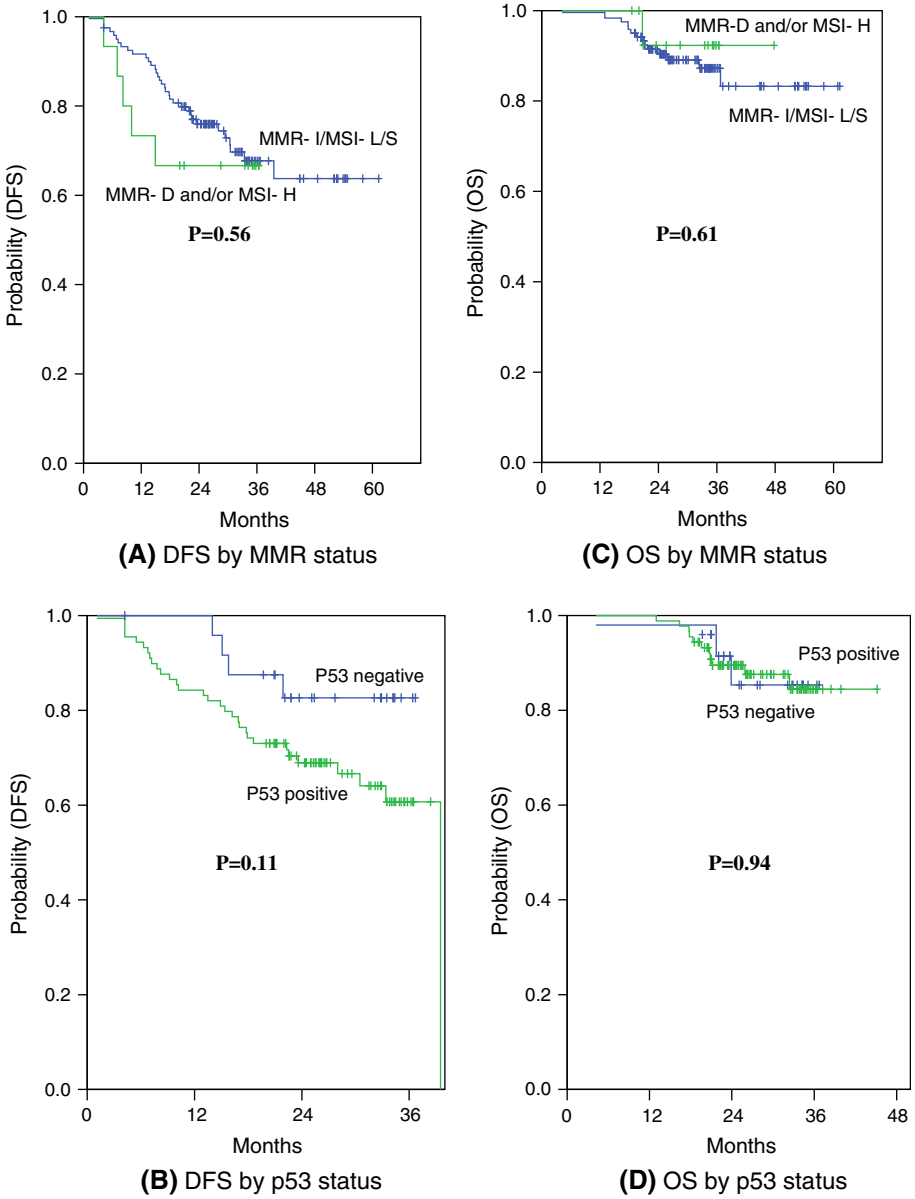

Fig. 1 **a**, **b** Disease-free survival (DFS) and **c**, **d** overall survival (OS) by genotyping, IDC, MMR status, and p53 status

status in terms of DFS and OS (Table 5). This same analysis was conducted for stage II and III with genotyping and IHC agreement ($n = 94$). Among these patients, DFS and OS of patients with MSI-H/MMR-D tumors were no different compared with those with MSI-L/S/MMR-I.

Discussion

This study represents first study to evaluate the efficacy of adjuvant chemotherapy containing 5-FU and oxaliplatin (FOLFOX), which is the standard postoperative regimen in colon cancer with MSI-H/MMR-D. In a pooled analysis of randomized chemotherapy trials, patients with colon cancer who had an inability to correct certain genetic alterations did not benefit from 5-FU-based chemotherapy [31]. Recently, the equalization of outcome for MSI-H and non-MSI-H tumors treated by irinotecan (CPT-11) and 5-FU as adjuvant chemotherapy regimen has been reported and implicated that the addition of irinotecan might overcome a negative impact of 5-FU on MSI-H tumors in adjuvant setting [6]. Similarly, we also observed no difference in DFS or OS between the MSI-H and non-MSI-H groups after postoperative FOLFOX chemotherapy.

Experimental evidence has identified links between MMR-D and cytotoxic drug resistance for alkylating agents [7, 18, 29]. Selection for cisplatin resistance in several human cancer cell lines resulted in loss of expression of the MMR proteins MLH1 and MSH2 in most (90%) cell lines, implicating the MMR system in cisplatin resistance [7].

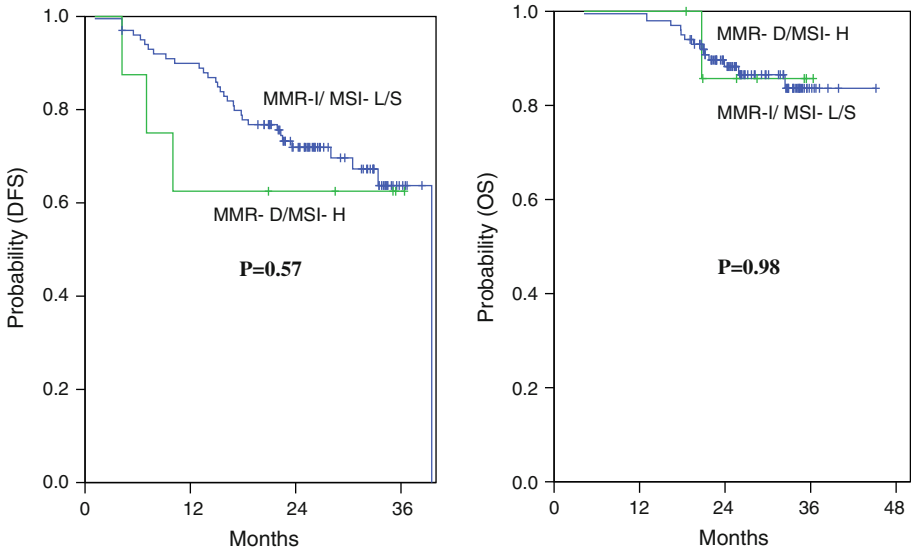

Fig. 2 Disease-free survival (DFS) and overall survival (OS) between MSI-H/MMR-D and MSI-L/S/MMR-I tumors in patients with genotyping/IHC agreements ($N = 108$)

Table 5 Univariate analysis of DFS and OS in relation to genotyping, IHC, MMR status, and p53 in stage II and III

	3-year DFS		HR (95% CI)	3-year OS		HR (95% CI)
	Stage II and III (%)	*P*		Stage II and III (%)	*P*	
Genotyping						
MSI-H	66.7	0.36	0.62 (0.21–1.78)	90.9	0.99	0.99 (0.13–7.91)
MSI-L/S	73.1			90.5		
IHC						
MMR-D	63.6	0.23	0.52 (0.18–1.53)	88.9	0.95	0.94 (0.12–7.50)
MMR-I	72.6			87.6		
MMR status						
MMR-D and/or MSI-H	66.7	0.27	0.58 (0.22–1.53)	82.3	0.87	1.20 (0.15–9.50)
MMR-I and MSI-L/S	73.5			90.4		
P53 status						
Positive	67.6	0.19	0.45 (0.14–1.51)	87.2	0.96	0.97 (0.20–4.65)
Negative	85.6			88.8		

Cisplatin-sensitive cell lines and human biopsies are hypermethylation of the promoters of only one MLH1 alleles, whereas resistant cell lines all exhibit hypermethylation of the promoters of both MLH1 alleles [34, 35]. Treatment of cisplatin-resistant cell lines with 5-azacytidine, a methylation inhibitor, resulted in reexpression of MLH1 and consequently increased sensitivity to cisplatin. Whereas MMR is clearly involved in cisplatin activity, in vitro and preclinical experiments have shown that MLH1-, MSH2- and MSH6-deficient cells, which are resistant to cisplatin, are nonetheless susceptible to oxaliplatin and that defects in MMR are associated with a modest to moderate level of resistance to cisplatin but not to oxaliplatin [10, 11].

In line with previous preclinical data, poorer survival for MLH following 5-FU chemotherapy was not observed in our patient cohort. There were no significant differences according to genotyping, IHC, MMR status and p53 status in terms of DFS and OS of patients with colon cancer who underwent adjuvant FOLFOX after curative resection. Substantial data, largely from retrospective trials, indicate that patients with MMR-D/MSI-H colon cancers achieve improved survival when compared with those with MMR-I/MSI-L/S tumors [15, 30]. Some studies suggest that FU adversely affects outcome for MSI-H tumors, and the preponderance of data indicate that FU is at best ineffective in this form of disease [5, 23, 31]. Our results represented that the MMR status was not significantly associated with outcomes to FOLFOX as adjuvant chemotherapy in colon cancer patients with R0 resection. Moreover, the probability of 3-year disease-free survival and overall survival in patients with MMR-D/MSI-H colon cancers who were treated with FOLFOX as adjuvant chemotherapy were 66.7 and 92.3%. This result looks like superiority when compared to that of previous trials for adjuvant FU/LV in stage

colon cancer irrespective of microsatellite status. On the basis of this indirect comparison, we reported that adding oxaliplatin in adjuvant chemotherapy may overcome negative impact of 5-FU on colon cancers with MSI-H/MMR-D. However, due to inherent bias from retrospective analysis with no comparator arm of 5FU alone and rather small sample size, prospective validation of prognostic capacity of MMR status should be performed.

About 78% (89/114) patients were positive for the p53 protein expression. P53 expression was known to occur more often in N0-1 and well/moderately differentiated tumors [26, 27, 32]. In this study, the status of p53 expression did not have significant impact for DFS and OS in patients with colon cancer treated by adjuvant FOLFOX, which is consistent with previous studies [27]. The largest study on p53 overexpression in early stage colorectal cancer demonstrated no significant prognostic value of p53 protein overexpression in 465 colon cancer samples [3]. Thus, p53 expression status may not be an indicator of prognosis in colon cancer.

Although this study is limited from retrospective analysis and small number of patients and rather short follow-up duration, our results suggest a possible sensitivity to oxaliplatin for MSI-H/MMR-D colon cancer.

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