Molecular prognostic factors in rectal cancer treated by preoperative chemoradiotherapy

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Abstract. The present study evaluated the expression of p53, pRb, hMLH1 and MDM2 prior to preoperative chemoradiotherapy (CRT) in patients with rectal cancer, and attempted to determine any correlation with treatment outcome. Forty-five patients with available pretreatment biopsy tissues and who received preoperative CRT were enrolled in this study. Preoperative CRT consisted of a median 50.4 Gy and 2 cycles of concurrent administration of 5-fluorouracil + leucovorin. Surgery was performed approximately seven weeks after CRT. Protein expression in formalin-fixed paraffin-embedded biopsy specimens was assessed by immunohistochemistry. A positive expression of p53, pRb, hMLH1 and MDM2 was found in 40, 46.7, 40 and 66.7% of the tissue specimens, respectively. The 5-year overall (OS), disease-free (DFS) and locoregional recurrence-free survival (LRFS) rates for patients included in the study were 71.3, 66.1 and 60.9%, respectively. p53 expression presented a significantly different OS (positive vs. negative, 45.8 vs. 86.2%; p=0.02). However, the expression of pRb, hMLH1 and MDM2 was not significant for OS. The expression of p53 was a borderline significant prognostic factor for DFS and for LRFS. Age, p53 and MDM2 expression were significant factors in the multivariate analysis performed for OS with 12 covariates, including 8 clinicopathological parameters and 4 proteins. No significant factor affected DFS or LRFS in the multivariate analysis. We suggest that the expression of p53 is a potential marker of survival. Determinations of this protein expression may be useful for selecting candidates from rectal cancer patients for more tailored treatment.

Introduction

Preoperative chemoradiotherapy (CRT) has been used extensively for locally advanced rectal cancer (LARC).

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As compared with conventional postoperative CRT, many studies have reported improved local tumor control, increased respectability, decreased toxicities and a higher sphincter-preservation rate (1-4). Preoperative CRT is also able to reduce tumor volume significantly. Kim *et al* have reported the mean percent volume reduction rate of primary tumors as 66.5% using magnetic resonance volumetry performed before and after CRT (5). Although the down-staging rate of primary tumors is approximately 50-60% for the use of preoperative CRT (1,2,4), a significant proportion of patients did not achieve a sufficient response to preoperative CRT, even with a long interval from CRT to surgery. To identify patients that may achieve a favourable response to treatment and who are at risk for treatment failure, as well as to predict survival prognosis, a number of molecular markers were recently investigated in patients with LARC. These molecular markers would allow for the delivery of individualized treatment regimens. Studies were performed on LARC patients treated with preoperative CRT. These studies have reported that expression patterns of biomarkers including Bax, epidermal growth factor receptor, p21, Ki-67 and caveolin-1 were correlated with response to CRT, local tumor control or survival (6-10).

We investigated the expression of molecular markers including p53, pRb, hMLH1 and MDM2. p53 activates genes that induce apoptosis in DNA-damaged cells (11). In view of survival prognosis, no studies have reported on a significant correlation of p53 expression, especially for the use of preoperative CRT, while studies with the use of preoperative radiotherapy alone have reported that patients with a higher p53 expression had a shorter survival (12,13). pRb strongly binds E2F and regulates the cell cycle by inducing G1 arrest (14). Results are conflicting for the expression of pRb and survival prognosis in patients with colorectal cancer treated with surgery without the use of preoperative CRT (15,16). Furthermore, no study has reported on the clinical significance of pRb expression in patients treated with preoperative CRT. hMLH1 is a component of the DNA mismatch repair (MMR) system responsible for the detection and repair of DNA lesions, e.g., mismatches, small insertions and deletions (17,18). For LARC treated with preoperative CRT, conflicting findings in a few studies have been reported for the expression of hMLH1 and treatment response (19,20), but no correlation with survival has been reported (20). MDM2 is a murine double-minute 2 oncogene product that forms a

stable complex with wild-type and mutant p53 proteins (21). The MDM2 oncogene is amplified or overexpressed in many human types of cancer, and the overexpression of MDM2 is correlated with a poor prognosis (22,23). Concerning MDM2 expression in LARC patients treated with preoperative CRT, no studies reported a significant relationship with tumor response or survival (24,25).

Few reports exist regarding the significance of the bio-markers in patients treated with preoperative CRT for rectal cancer. In this study, we evaluated the expression of these bio-markers prior to preoperative CRT and attempted to correlate their expression with treatment outcome.

Materials and methods

Patients and pretreatment evaluation. Between March 2000 and May 2007, >100 patients with locally advanced rectal cancer received preoperative chemoradiotherapy at our institution. More than half of them received pretreatment pathological diagnosis at an outside referral hospital. Forty-five patients with available biopsy tissue specimens at our institution or nearby referral hospital were enrolled in the study. Pretreatment work-up included a complete history, physical examination, complete blood count, serum chemistry, determination of the carcinoembryonic antigen (CEA) level, chest radiography, an abdominal/pelvic CT scan and colonoscopy with biopsy. Informed consent was obtained from patients included in the study before treatment.

Treatment and follow-up. Radiation was delivered with 10-MV photons by the use of a three-field technique (posterior and both lateral fields). Radiotherapy was delivered 5 days/week, once/day, at a dose of 1.8 Gy/day. Pelvic radiotherapy consisted of a median dose of 43.2 Gy in 24 fractions over 5 weeks, which was followed by a median boost dose of 7.2 Gy, administered in 4 fractions to the primary tumor by the use of three fields. Chemotherapy consisted of 2 cycles of intravenous bolus 5-FU (500 mg/m^2/day) and leucovorin (20 mg/m^2/day) for 5 days each, with or without cisplatin on the sixth day. At approximately seven weeks after the completion of CRT, the patients underwent definitive surgery. Surgical management included the use of a sphincter preservation approach whenever possible, by use of the total mesorectal excision technique. Each patient was provided with follow-up including a complete blood count, serum chemistry and determination of the CEA level every 3 months. Chest radiography and an abdominal/pelvic CT scan were performed every 6 months in the first 2 years. Follow-up was then performed every 6 months thereafter.

Immunohistochemical staining procedure. Biopsy specimens were obtained via a pretreatment endoscopy with 1 sample in each case. Specimens were routinely fixed in 10% buffered formalin and were embedded in paraffin. After reviewing hematoxylin and eosin-stained slides, serial sections from each block were used for immunohistochemistry. We carried out immunohistochemical staining with the use of the Microprobe Immuno/DNA stainer (Fisher Scientific, Pittsburgh, PA, USA). Immunohistochemical staining with primary antibodies for p53 (BP53-12, diluted 1:50; Novocastra

Laboratories, Newcastle, UK), human pRb (clone Rb1, diluted 1:40; Dako, Glostrup, Denmark), hMLH1 (G168-15, diluted 1:100; Zymed Laboratories, South San Francisco, CA, USA) and human MDM2 (clone IF2, diluted 1:100; Oncogene Research Products, Cambridge, MA, USA) was performed by use of the avidin-biotin-peroxidase complex method. Sections were deparaffinized and placed in a microwave oven with a 2.1% citric acid buffer solution (pH 6.0) for 10 min to retrieve the antigens. Following microwave processing, the sections were incubated overnight at 4°C with primary antibodies. The use of a streptavidin-horseradish peroxidase detection system (Research Genetics, Huntsville, AL, USA) was then applied to capillary channels, followed by a 10-min incubation at 50°C. Reaction products were visualized with diaminobenzidine as a chromogen and slides were then counterstained with hematoxylin. Rectal cancer tissue strongly expressing p53 was used as a positive control for p53 and MDM2, while normal rectal epithelium adjacent to a tumor was used as a positive control for pRb and hMLH1. For negative controls, sections were treated similarly with omission of the primary antibody.

Assessment of p53, pRb, hMLH1 and MDM2 expression. Each slide was examined under the same magnification (x200, Vanox-S; Olympus, Tokyo, Japan) by one independent pathologist who moved the microscopic field randomly across specimens. Immunoreactivity was evaluated according to the frequency of positive nuclear staining. Semi-quantitative, 4-tier grading was based on the percentage of tumor cells that stained positive in the entire tumor boundary: 0, negative; 1, minimal (<10% staining); 2, moderately stained (10-50% staining) and 3, markedly stained (≥50% staining). Results were classified into two groups according to the number of positively stained cancer cells. Criteria for positive expression were defined as >50% staining of the tumor tissue for hMLH1 and p53 expression, >10% staining for pRb expression and >0% for MDM2 expression.

Statistical analysis. The relationship between the clinicopathological factors and protein expression was analyzed by use of the Chi-square or Fisher's exact test, as appropriate. Overall survival (OS) was defined as the time from the starting date of preoperative CRT until the date the patient succumbed to any cause or of the last follow-up; disease-free survival (DFS) until the date of any recurrence as a first event and loco-regional recurrence-free survival (LRFS) until the date of the first detection of recurrence in the pelvis. Survival outcomes were calculated by use of the Kaplan-Meier method. The prognostic value of the protein expression was evaluated by use of the log-rank test for univariate analysis and the Cox proportional hazards model for multivariate analysis. P<0.05 was considered as statistically significant. Statistical analyses were conducted by use of the SPSS version 17.0 statistical software (SPSS, Chicago, IL, USA).

Results

Immunohistochemical analysis of p53, pRb, hMLH1 and MDM2 expression. A positive expression of p53, pRb, hMLH1 and MDM2 were found in 40, 46.7, 40 and 66.7% of the tissue specimens, respectively. Fig. 1 shows representative staining
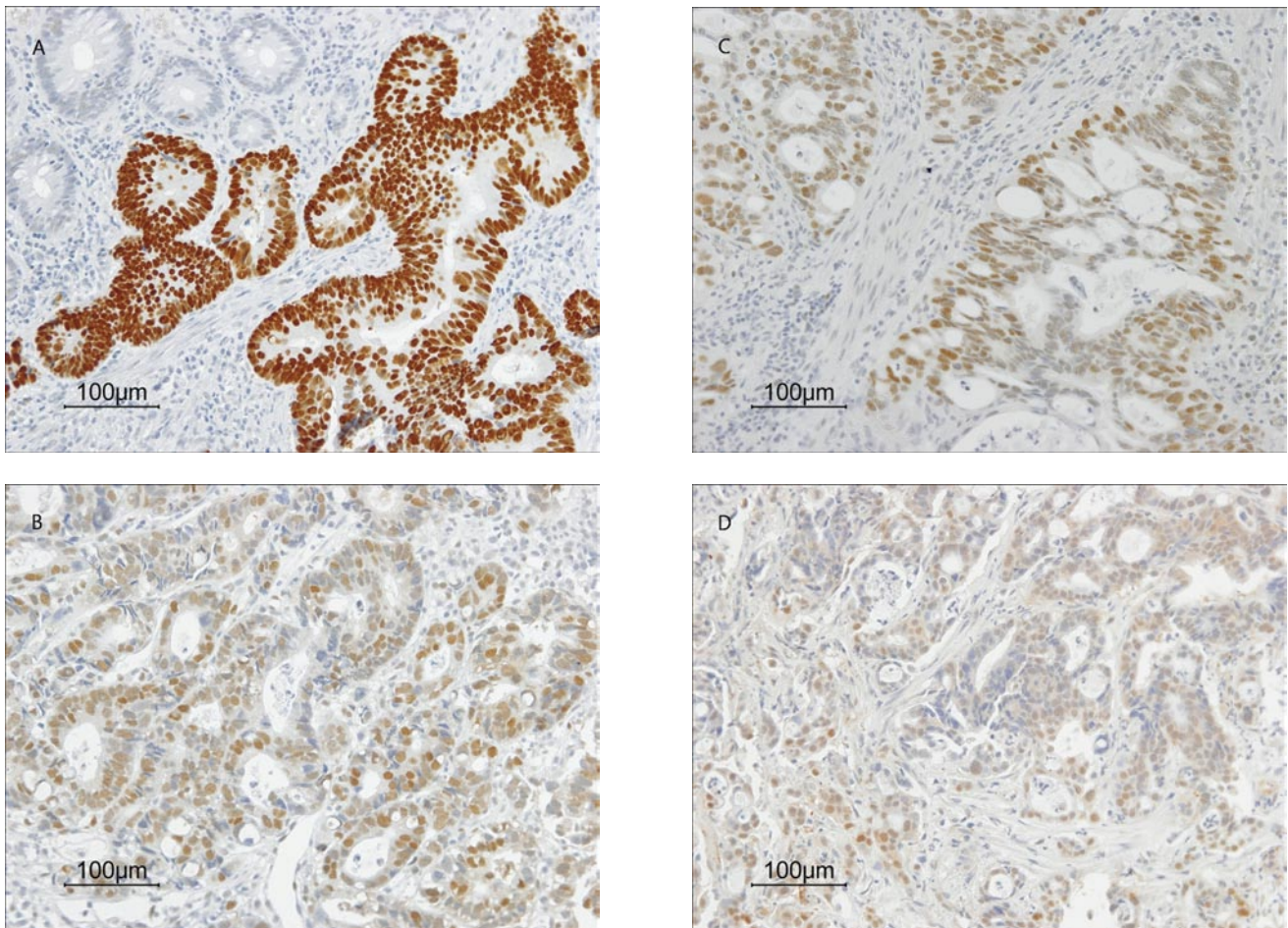
Figure 1. Positive immunohistochemical staining of (A) p53 expression, (B) pRb expression, (C) hMLH1 expression and (D) MDM2 expression. Original magnification, x200.

results of these proteins. Correlations between protein expression and patient clinicopathological factors are listed in Tables I and II. No significant correlation was observed for expression and clinicopathological factors including gender, age, preoperative clinical stage, postoperative pathological stage or primary tumor response. However, patients with a negative pRb expression achieved more complete tumor response and were of a younger age, with a marginal significance.

Relationship between protein expression and survival. After preoperative CRT, pathological complete response (CR) of primary tumors occurred in 10 patients (22.2%). Pathological staging was as follows: pT0N0 in 7 patients (15.6%), pT0N1-2 in 3 patients (6.6%), pT1-2N0 in 7 patients (15.6%), pT3-4N0 in 18 patients (40%) and pT2-3N1-2 in 10 patients (22.2%), including 1 patient with pT2N1M1. The follow-up range of the patients was 11-107 months (median 38.5). The 5-year OS, DFS and LRFS rates for the patients inclued in the study were 71.3, 66.1 and 60.9%, respectively. The 5-year OS rates for pathological stages 0 (n=7), I (n=7), II (n=13), III (n=13) and IV (n=1) were 100, 42.9, 73.8, 53.9 and 0%, respectively (p=0.11). Only p53 expression was a significant prognostic factor affecting OS in the univariate analysis (positive vs. negative expression, 45.8 vs. 86.2%; p=0.02; Fig. 2A). The expression of hMLH1 (positive vs. negative, 71.2 vs. 71.8%; p=0.87), MDM2 (posi-

tive vs. negative, 72.7 vs. 65%; p=0.31) and pRb (positive vs. negative, 72.9 vs. 70%; p=0.92) did not affect OS significantly. The 5-year DFS rates for pathological stages 0, I, II, III and IV were 100, 57.1, 68.8, 53.8 and 0%, respectively (p=0.000). p53 expression was a borderline significant prognostic factor affecting DFS (positive vs. negative, 50 vs. 77%; p=0.06; Fig. 2B). The expression of hMLH1 (positive vs. negative, 54.3 vs. 74.1%; p=0.24), MDM2 (positive vs. negative, 69.7 vs. 59.3%; p=0.49) and pRb (positive vs. negative, 59.3 vs. 70.8%; p=0.48) did not affect DFS significantly. The 5-year LRFS rates for pathological stages 0, I, II, III and IV were 85.7, 47.6, 69.7, 41 and 100%, respectively (p=0.31). p53 expression was a borderline significant prognostic factor affecting LRFS (positive vs. negative, 45.1 vs. 70%; p=0.07; Fig. 2C). The expression of hMLH1 (positive vs. negative, 69.9 vs. 56.6%; p=0.79), MDM2 (positive vs. negative, 58.2 vs. 58.7%; p=0.50) and pRb (positive vs. negative, 58.8 vs. 62%; p=0.85) did not affect LRFS significantly.

Relationship of the clinicopathological parameters, protein expression and survival by multivariate analysis. Multivariate analysis was performed for OS with the use of 12 covariates. The covariates included age (≤55 vs. >55 years), gender, clinical stage (I + II vs. III), tumor location from the anal verge (<5 vs. ≥5 cm), interval between radiotherapy and surgery

Table I. Clinicopathological characteristics of p53 and pRb protein expression.

Characteristics	p53		P-value	pRb		P-value
	Positive	Negative		Positive	Negative	
Gender			0.72			1.00
Male	15	21		17	19	
Female	3	6		4	5	
Age (years)			0.39			0.08
≤55	9	10		6	13	
>55	9	17		15	11	
cStage[a]			0.90			0.23
I + II	7	10		6	11	
IIIb + IIIc	11	17		15	13	
pStage[b]			0.38			0.12
0	1	6		1	6	
I + II	11	13		14	10	
III + IV	6	8		6	8	
Response			0.14			0.06
CR[c]	2	8		2	8	
Non-CR	16	19		19	16	

[a]Preoperative clinical stage; [b]postoperative pathological stage; [c]complete response of primary tumor.

Table II. Clinicopathological characteristics of hMLH1 and MDM2 protein expression.

Characteristics	hMLH1		P-value	MDM2		P-value
	Positive	Negative		Positive	Negative	
Gender			0.72			0.70
Male	15	21		23	13	
Female	3	6		7	2	
Age (years)			0.81			0.14
≤55	8	11		15	4	
>55	10	16		15	11	
cStage[a]			0.45			0.13
I + II	8	9		9	8	
IIIb + IIIc	10	18		21	7	
pStage[b]			0.41			0.26
0	2	5		3	4	
I + II	12	12		16	8	
III + IV	4	10		11	3	
Response			0.36			0.44
CR[c]	3	7		6	4	
Non-CR	15	20		24	11	

[a]Preoperative clinical stage; [b]postoperative pathological stage; [c]complete response of primary tumor.

(≤6 vs. >6 weeks), sphincter-saving surgery or not, primary tumor response (CR or non-CR), pathological stage (0, I, II, III and IV) and expression of the four proteins. Age, MDM2 and p53 expression were significant factors affecting OS (Table III). No significant factor was determined for DFS or LRFS by multivariate analysis.
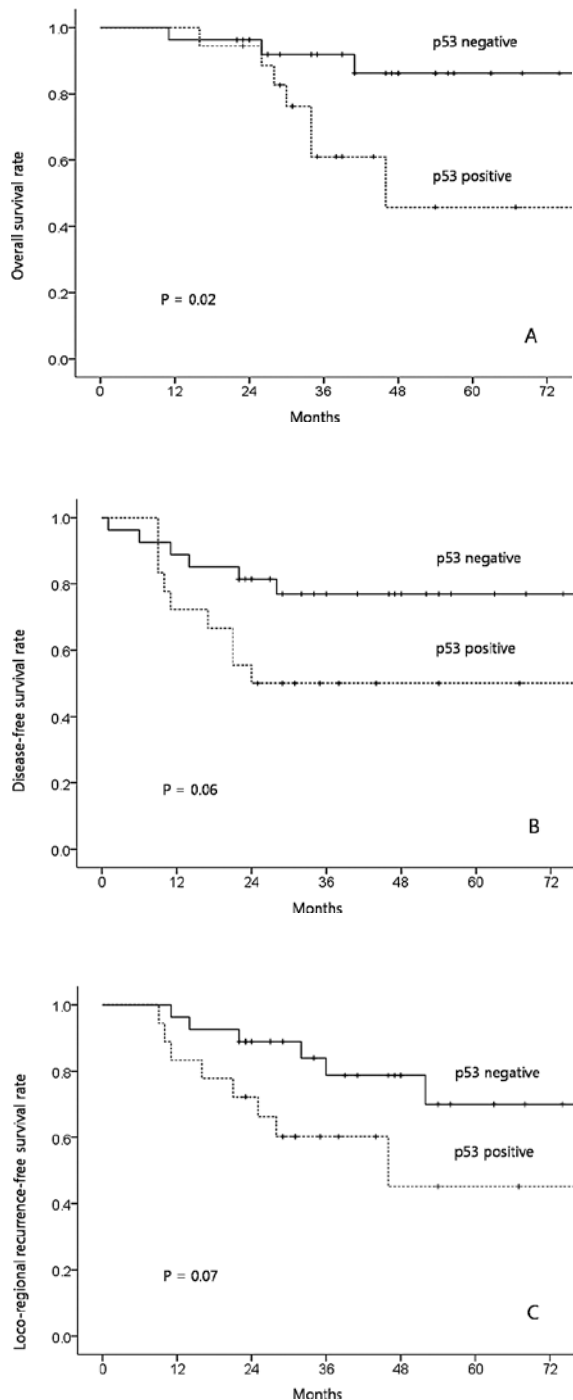
Figure 2. Univariate Kaplan-Meier survival analyses. (A) Overall, (B) disease-free and (C) loco-regional recurrence-free survival according to the pattern of p53 expression.

Discussion

To identify patients that may achieve favorable response to treatment and are at risk for treatment failure, as well as to predict survival prognosis, a number of molecular markers were recently investigated in LARC patients. These molecular markers were examined to determine whether they would allow for the delivery of individualized treatment regimens. Epidermal growth factor expression has been associated with a poor response to preoperative chemoradiotherapy and worse DFS (7,8). Rau et al reported that the expression of p21 appeared to predict worse DFS, while that of Ki-67 predicted better DFS in patients treated with neoadjuvant CRT (9). From the four proteins studied, MDM2 and p53 expression was found to be significantly related to OS. In addition, a negative pRb expression was associated with a more CR. Although p53 expression was found to have borderline significance for DFS or LRFS, we consider that the significance of p53 expression for DFS or LRFS may have improved had the analysis been conducted on a larger number of patients with a longer follow-up period.

The p53 gene facilitates DNA repair or apoptosis in response to DNA damage, whereas p53 gene mutations result in functional abnormalities that lead to radioresistance (26,27). Shimoji et al reported that p53 immunoreactivity in esophageal carcinoma specimens, obtained prior to preoperative radiotherapy, was significantly correlated with radioresistance (27). In LARC patients treated with preoperative CRT, some studies have reported a poorer response in tumors with a higher p53 immunohistochemical staining of pretreatment biopsy specimens (19,28), while other studies did not determine any correlation (24,29). In the present study, pathological complete remission was reported in 22.2% of the patients. However, our study failed to show any statistically significant correlation between baseline p53 expression and the pathological response to CRT, although a trend for a higher pathological CR rate was observed in tumors negative for p53 expression (Table I). Concerning survival prognosis, studies of preoperative radiotherapy alone have reported that patients with a higher p53 expression showed shorter survival (12,13). However, other studies have reported no relationship in LARC patients treated with preoperative CRT (20,30). Our results showed that p53 was an independent prognostic factor affecting OS and a marginally significant factor for DFS or LFRS.

pRb strongly binds E2F and regulates the cell cycle by inducing G1 arrest (14). pRb is able to act as a survival factor in colonic epithelial cells by suppressing apoptosis.The

Table III. Cox proportional hazards model for overall survival.

Characteristics	HR	95% CI	P-value
Age (>55 vs. ≤55 years)	58.109	3.864-873.841	0.003
p53 (positive vs. negative)	15.347	2.209-106.618	0.006
MDM2 (positive vs. negative)	0.073	0.010-0.517	0.009
Sphincter-saving surgery (no vs. yes)	4.187	0.847-20.703	0.079

HR, hazard ratio; CI, confidence interval.

overexpression of pRb in colorectal tumor cells can cause a loss of sensitivity to apoptotic signaling, resulting in aberrant cell survival and resistance to therapy (31). Our study showed that a trend for a higher pathological CR rate was observed in tumors with a negative pRb expression (Table I). Although the difference was not statistically significant, our results are in accordance with the observation that the overexpression of pRb may cause a loss of sensitivity to apoptotic signaling. It remains debatable whether pRb expression is of clinical significance in various types of cancer (32,33). At resectable stages, most colorectal cancer types demonstrate an aberrant expression of pRb and/or p16. One study with surgery alone has reported that the aberrant expression of pRb and p16, alone and in combination, is associated with poor prognosis in patients with colorectal cancer (15), while other studies have reported no correlation with survival prognosis (16,34). Our study demonstrated that pRb expression had no prognostic significance affecting survival, but had a marginal significance regarding preoperative CRT response, i.e., the negative pRb expression group showed a more CR of the primary tumor.

hMLH1 is a component of the DNA MMR system, which participates in DNA checkpoints and sends apoptotic signals (35). It is controversial as to whether radiation sensitivity is affected by the microsatellite instability or MLH1 status. Davis *et al* reported that MLH1-deficient human colon carcinoma cells show lower survival as compared to MMR-corrected human colon carcinoma cells because of a deficiency in the G2-M cell cycle checkpoint arrest after radiation treatment (36). In LARC patients treated with preoperative CRT, a few studies have shown conflicting results regarding response to CRT. Charara *et al* reported that patients with a lower expression of hMLH1 or higher microsatellite instability showed better treatment response (19), while Bertolini *et al* reported that patients treated with preoperative CRT and a positive hMLH1 expression had a higher CR rate (24.3 vs. 9.4%; p=0.055), but no correlation with survival (20). We previously reported that a low hMLH1 expression was found to be associated with poor prognosis in patients treated with definitive CRT for esophageal cancer (37). However, in the present study of LARC patients, we did not determine any significance of hMLH1 expression.

MDM2 is a murine double-minute 2 oncogene product that forms a stable complex with wild-type and mutant p53 proteins (21). MDM2 is responsible for shuttling p53 from the nucleus to the cytoplasm, promoting p53 degradation by proteasomes (38). MDM2 also binds with pRb, thus inhibiting the growth regulatory function of pRb in a p53-independent manner (39). The MDM2 oncogene is amplified or overexpressed in many human types of cancer and has a role in tumor growth through p53-dependent and -independent mechanisms. Moreover, MDM2 overexpression is correlated with a poor prognosis in many cancer types (22,23). Kondo *et al* reported that disruption of the p53/MDM2/p14ARF pathway may frequently participate in colonic carcinogenesis. Moreover, the MDM2 expression status may be a factor in the prediction of potential invasion and the presence of liver metastases for colorectal carcinomas (22). Forslund *et al* found MDM2 to be amplified in 9% of the 284 colorectal cancers analyzed and MDM2 gene amplification was significantly correlated with advanced tumor stage (23). These investigators suggested that MDM2

is a promising target for cancer therapy in colorectal cancer for the use of small molecule MDM2 antagonists that can inhibit p53-MDM2 binding. Concerning the clinical significance of MDM2 expression in LARC, one study reported that MDM2 expression had no relationship with tumor response to preoperative CRT (24), while another study reported no correlation with survival in patients treated only with surgery (25). In the present study, a positive MDM2 expression was detected in 30 cases (66.7%), showing a favorable prognosis with statistical significance in the multivariate analysis for OS. However, we did not consider its clinical significance, since MDM2 expression was not significant for any survival in the univariate analysis. Nevertheless, a further study is needed to elucidate whether the MDM2 oncoprotein is able to counteract or offset the poor prognostic role of mutant p53 protein, as well as what possible mechanism exists between them.

In conclusion, a negative p53 expression was found to be an independent prognostic marker for improved OS in this study. Patients with a negative pRb expression had a more CR to preoperative CRT. We suggest that the expression of p53 is a potential marker of survival that may be useful for selecting candidates from LARC patients for more tailored treatment.

Acknowledgements

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