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Association of p53 codon 72 polymorphism with liver metastases of colorectal cancers positive for p53 overexpression*

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Abstract: Objective: To evaluate the association between p53 codon 72 polymorphism (R72P) and the risk of colorectal liver metastases. Methods: The p53 R72P genotype was identified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method in 78 consecutive colorectal cancer patients with liver metastases and 214 age- and sex-matched cases with nonmetastatic colorectal cancer. Results: The R allele of the p53 R72P polymorphism was more frequently found in metastatic cases than in nonmetastatic cases (P=0.075). Carriers of the 72R allele had a 2.25-fold (95% *CI* (confidence interval)=1.05~4.83) increased risk of liver metastases. On the stratification analysis, 72R-carrying genotype conferred a 3.46-fold (95% *CI*=1.02~11.72) and a 1.05-fold (95% *CI*=0.36~3.08) increased risk of liver metastases for p53 overexpression-positive and negative colorectal cancers, respectively. Conclusion: These results demonstrate for the first time that the 72R allele of the p53 polymorphism has an increased risk for liver metastases in colorectal cancers positive for p53 overexpression.

Key words: Colorectal cancer, p53, Genetic polymorphism, Liver metastases, Overexpression
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INTRODUCTION

Liver metastasis is the most common cause of colorectal cancer-related mortality (Burke and Allen-Mersh, 1996). It is now known that multiple risk factors contribute to the development of liver metastases, including sex, age, colorectal tumor features, and a variety of genetic abnormalities involved in invasion and metastases (Adachi *et al*., 1999; Ogawa *et al*., 2005; Ghadjar *et al*., 2006; Kuramochi *et al*., 2006; Manfredi *et al*., 2006; Shinji *et al*., 2006; Herynk *et al*., 2007; Lin *et al*., 2007). Here, we investigated the effect of a common p53 polymorphism (R72P) on colorectal liver metastases in a Chinese population.

p53 tumor suppressor gene is a potential candidate for modulating the risk of colorectal liver metastases. Several studies showed that an increased incidence of p53 mutation and related overexpression of the gene are associated with liver metastases of colorectal tumors (Kastrinakis *et al*., 1995; Kimura *et al*., 1996; Kang *et al*., 1997), suggesting a role for p53 in the establishment of colorectal liver metastases. It has been proposed that wild-type p53 has an antiangiogenic effect, possibly by up-regulation of the angiogenesis inhibitor thrombospondin-1

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(Dameron *et al*., 1994). However, mutant p53 was found to stimulate vascular endothelial growth factor production (Kieser *et al*., 1994). Moreover, a positive correlation between p53 mutations and intratumoral microvessel density was found in colorectal cancers (Vermeulen *et al*., 1996; Kang *et al*., 1997; Liang *et al*., 2004) and liver metastases (de Jong *et al*., 2005). These data suggest that in primary colorectal cancers, p53 mutations are associated with a pro-angiogenic state and, thus, with a higher chance of liver metastases.

p53 gene has a common polymorphism that results in either arginine (R) or proline (P) at codon 72. Functional differences between the two polymorphic forms have been described previously (Lanham *et al*., 1998; Thomas *et al*., 1999; Dumont *et al*., 2003). The 72R allele of mutant p53 has been found to have greater ability to bind p73, neutralizing p73-induced apoptosis, when compared to the mutant 72P allele (Marin *et al*., 2000). Moreover, the 72R allele is preferentially mutated and retained in various human tumors arising in RP germline heterozygotes (Marin *et al*., 2000; Kawaguchi *et al*., 2000; Tada *et al*., 2001; Langerod *et al*., 2002; Papadakis *et al*., 2002; Anzola *et al*., 2003; Schneider-Stock *et al*., 2004a; Hsieh *et al*., 2005). Recently a positive association of the 72R allele with p53 overexpression has been reported in esophageal cancer tissue (Lee *et al*., 2006). Furthermore, the 72R allele has been associated with increased tumor stages in urinary tract cancers (Furihata *et al*., 2002) and colorectal cancers (Schneider-Stock *et al*., 2004b; Perez *et al*., 2006). These results indicate that the p53 P and R alleles at 72 codon are functionally distinct, which may influence cancer progression and metastases. To the best of our knowledge, data on association of the p53 R72P polymorphism with colorectal liver metastases are presently not available.

In the present study, we examined the association between p53 R72P polymorphism and the risk of colorectal liver metastases in a case-case study of 78 patients with colorectal liver metastases and 214 age- and sex-matched cases with nonmetastatic colorectal cancer in a Chinese population. We also examined whether the potential association of p53 R72P polymorphism with the risk of colorectal liver metastases differs according to p53 overexpression status in colorectal cancer tissue.

MATERIALS AND METHODS

Patients

This study included 78 consecutive colorectal cancer patients with liver metastases and 214 age- and sex-matched cases with nonmetastatic colorectal cancer as a control. All patients were recruited between 2003 and 2005 at No. 113 Hospital of People's Liberation Army and Lihuili Hospital in Ningbo, China. Final diagnosis of all colorectal adenocarcinoma was confirmed by histopathological examination. Diagnosis and presence of liver metastases were based on: (1) positive findings on cytological or pathological examination, and/or (2) positive images on angiogram, ultrasonography, computed tomography (CT), and/or magnetic resonance imaging (MRI). Cases with liver metastases diagnosed within 3 months after the initial diagnosis of colorectal cancer were considered to have synchronous liver metastases.

Data on all patients were obtained from medical records, pathology reports and personal interviews with the patients. The data collected include sex, age, and colorectal tumor features such as tumor size, tumor location, histological grade, depth of invasion, and lymph node metastasis. Information on p53 overexpression was available from 239 patients (76 with liver metastases). Fifty-one cases in nonmetastases group and 2 in metastases group did not undergo surgery because of economic or other reasons, and their colorectal cancer diagnosis was based only on biopsy. Therefore, these cases were excluded from analysis of p53 overexpression owing to too small cancer tissues. Immunohistochemical staining for p53 was performed on paraffin-embedded sections using mouse anti-human p53 monoclonal mutant protein (DO-7, Dako, Denmark). Samples were considered positive when 10% of the cancer cells were positive for p53 staining. Each subject provided written consent to participate in the study and to donate a 5 ml blood sample for genetic testing. The research protocol was approved by the Institutional Review Board of two participant hospitals (No. 113 Hospital of People's Liberation Army and Lihuili Hospital in Ningbo, China).

Genotyping

Genomic DNA was extracted from peripheral blood lymphocytes by proteinase K digestion and

phenol/chloroform extraction. Molecular analysis for the p53 R72P genotypes was performed as previously described (Zhu *et al*., 2005). Briefly, it involved a combination of polymerase chain reaction (PCR) and digestion with restriction endonuclease BstU I, followed by non-denaturing polyacrylamide gel electrophoretic analysis. PP homozygotes were represented by a DNA band of 199 bp in size, whereas RR homozygotes were represented by DNA bands of 113 and 86 bp. RP heterozygotes displayed a combination of both alleles (199, 113, and 86 bp). Genotyping results were further confirmed by direct DNA sequencing.

Statistical analysis

Differences in sex, age, and colorectal tumor features (including tumor size, tumor location, histological grade, depth of invasion, lymph node metastasis, and p53 overexpression) between groups with and without liver metastases were evaluated using the χ^2-test. The association between p53 R72P and the risk of colorectal liver metastases was estimated by computing the odds ratios (ORs) and their 95% confidence intervals (CIs) from multivariate logistic regression analyses with adjustment for potential risk factors. Hardy-Weinberg equilibrium was tested using the asymptotic Pearson's χ^2-test. All statistical analyses were conducted using the Stata 9.0 (Stata Corporation, College Station, TX, USA) statistical package. All tests were two-sided at the 0.05 significance level.

Table 1 Distribution of selected variables in metastases and nonmetastases patients

Variables	Nonmetastases, *n*	Metastases, *n*	*P*[a]
Sex			0.915
Female	70 (32.7%)	25 (32.1%)	
Male	144 (67.3%)	53 (67.9%)	
Age			0.931
≤60 years	114 (53.3%)	42 (53.8%)	
>60 years	100 (46.7%)	36 (46.2%)	
Colorectal tumor size			0.180
≤4 cm	99 (46.3%)	43 (55.1%)	
>4 cm	115 (53.7%)	35 (44.9%)	
Colorectal tumor location[b]			0.299
Proximal colon	72 (34.1%)	31 (40.8%)	
Distal colorectum	139 (65.9%)	45 (59.2%)	
Colorectal tumor grade[b]			0.168
Well	43 (20.4%)	8 (10.7%)	
Moderately	109 (51.6%)	43 (57.3%)	
Poorly	59 (28.0%)	24 (32.0%)	
Depth of tumor invasion[b,c]			0.004
T1 or T2	71 (33.8%)	13 (16.7%)	
T3 or T4	139 (66.2%)	65 (83.3%)	
Lymph node metastasis[b]			<0.001
Negative	131 (63.9%)	21 (30.9%)	
Positive	74 (36.1%)	47 (69.1%)	
p53 overexpression[b]			0.107
Negative	89 (54.6%)	33 (43.4%)	
Positive	74 (45.4%)	43 (56.6%)	

[a]χ^2-test was used to test statistically significant differences between groups; [b]Numbers not add up to the total, owing to missing data; [c]Depth of tumor invasion corresponds to the T (tumor) description according to the TNM (tumor-nodes-metastasis) classification

RESULTS

The selected characteristics of the colorectal cancer patients are shown by metastases/nonmetastases status in Table 1. Distributions of sex, age, tumor location, tumor size, histological grade and p53 overexpression were not significantly different in patients with and without metastases. The patients with liver metastases were more likely to have deeper tumor invasion and lymph node metastasis compared with those without metastases.

Genetic data are summarized in Table 2. The p53 R72P genotype distributions in both metastases and nonmetastases patients were in Hardy-Weinberg equilibrium (P>0.05). The 72R allele was found more often in patients with metastases than in those without metastases, although the results did not reach statistical significance (P=0.075). When compared with PP homozygotes, the adjusted ORs of colorectal liver metastases were 2.38 (95% *CI*=1.08~5.24, P=0.031) for RP heterozygotes, 1.94 (95% *CI*=0.77~4.91, P=0.160) for RR homozygotes, and 2.25 (95% *CI*=1.05~4.83, P=0.038) for carriers of the 72R allele (RP or RR genotype).

The p53 R72P genotype distributions in the metastases and nonmetastases stratified by p53 overexpression in colorectal cancer tissue are shown in Table 3. The risk of liver metastases associated with the 72R-carrying genotype appeared to be stronger in patients with colorectal tumors overexpressing p53 (*OR*=3.46, 95% *CI*=1.02~11.72, P=0.046), whereas no significant association was found in patients whose tumors did not overexpress p53 (*OR*=1.05, 95% *CI*=0.36~3.08, P=0.935). A similar

Table 2 Association of p53 R72P polymorphism with risk of colorectal liver metastases

p53 R72P	Nonmetastases, *n*	Metastases, *n*	*OR*[a]	*P*[a]
PP	60 (28.0%)	11 (14.1%)	1.00	
RP	105 (49.1%)	47 (60.3%)	2.38 (95% *CI*=1.08~5.24)	0.031
RR	49 (22.9%)	20 (25.6%)	1.94 (95% *CI*=0.77~4.91)	0.160
R allele	203 (47.4%)	87 (55.8%)		
PP	60 (28.0%)	11 (14.1%)	1.00	
R carriers	154 (72.0%)	67 (85.9%)	2.25 (95% *CI*=1.05~4.83)	0.038

[a]Adjusted for the depth of invasion and lymph node metastasis

Table 3 Risk of colorectal liver metastases associated with the p53 R72P polymorphism stratified by p53 overexpression

p53 overexpression	p53 R72P	Nonmetastases, *n*	Metastases, *n*	*OR*[a]	*P*[a]
Positive	PP	20 (27.0%)	4 (9.3%)	1.00	
	R carriers	54 (73.0%)	39 (90.7%)	3.46 (95% *CI*=1.02~11.72)	0.046
Negative	PP	18 (20.2%)	7 (21.2%)	1.00	
	R carriers	71 (79.8%)	26 (78.8%)	1.05 (95% *CI*=0.36~3.08)	0.935

[a]Adjusted for the depth of invasion and lymph node metastasis

magnitude of the ORs was observed when the patients were stratified by sex, age, tumor size, location, histological grade, depth of invasion, and lymph node metastasis (data not shown).

DISCUSSION

We found that colorectal cancer patients with the 72R-carrying genotype of the p53 R72P polymorphism had a significantly increased risk of liver metastases when compared with those with PP genotype, and that this risk-effect is related to p53 overexpression in colorectal cancer tissue. Our data demonstrate for the first time a statistically significant association between p53 R72P and the risk of colorectal liver metastases in patients whose tumors overexpress p53. In line with our results, Brooks *et al*.(2000) found that of the five metastatic vulval cancers, four expressed 72R p53 mutant proteins.

The exact underlying mechanism(s) responsible for our finding that the p53 72R allele has an increased risk for colorectal liver metastases in cancers positive for p53 overexpression still remains unclear. Several reports indicated that the 72R allele is preferentially mutated and retained in various human tumors (Kawaguchi *et al*., 2000; Marin *et al*., 2000; Tada *et al*., 2001; Langerod *et al*., 2002; Papadakis *et al*., 2002; Anzola *et al*., 2003; Schneider-Stock *et al*., 2004a; Hsieh *et al*., 2005), and is associated with the presence of p53 overexpression (Lee *et al*., 2006). In experiments, mutant 72R allele tended to block p73 function more effectively than mutant 72P isoform, which might lead to a higher tendency of p53 mutation in tumors (Marin *et al*., 2000). Thus the 72R allele may function as a mediator responsible for a progressive genetic instability, leading to a more malignant potential, including the capability of invasiveness and metastasization. Clinical response following cisplatin-based chemo-radiotherapy for advanced head and neck cancers was reported to be influenced by this polymorphism, cancers expressing 72R mutants having lower response rates than those expressing 72P mutants (Bergamaschi *et al*., 2003). This result indicates that the 72R allele may limit individual responsiveness to cancer therapy, thereby contributing to cancer progression and metastases. Additional functional studies are needed in order to elucidate the role of p53 R72P polymorphism in the development of colorectal liver metastases.

The limitations of the current study should be borne in mind. This is a limited study because of small sample size and imbalance of tumor characteristics between metastases and nonmetastases. In addition, some misclassifications of metastases status are inevitable because a proportion of the colorectal cancer patients already have undetectable liver micrometastases at the time of diagnosis of primary

tumor (Bosman, 1995). In the present study, cases with liver metastases diagnosed within 3 months after the initial diagnosis of colorectal cancer were considered to have synchronous liver metastases and classified into the metastases group. Another limitation is that no data about p53 mutation of primary colorectal tumors were available. Immunoreactivity of monoclonal antibodies to p53 is not considered to adequately predict the presence of p53 mutations (Coggi *et al*., 1997; Lee *et al*., 2006). However, this may not lead to a bias for a conclusion that concerns colorectal cancers positive for p53 overexpression, since p53 overexpression detected by immunohistochemistry is usually regarded as the presence of p53 mutation. We, therefore, report for the first time that the R allele of the p53 R72P polymorphism has an increased risk for colorectal liver metastases in cancers positive for p53 overexpression. Future studies are needed to further clarify the role of p53 codon 72 polymorphism in liver metastases of colorectal cancers.

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