Hirosaki Med. J. 56 : 69—78, 2005

ORIGINAL ARTICLE

PROGNOSTIC SIGNIFICANCE OF *P53* MUTATION IN SURGICALLY TREATED COLORECTAL CANCER: A PROSPECTIVE STUDY USING DIRECT SEQUENCING METHOD

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Abstract To evaluate the clinical significance of *p53* mutations, we analyzed the relationship of several clinicopathologic factors to the clinical outcomes in 131 colorectal cancer patients. Exons 5 to 9 of the *p53* gene were studied by the direct sequencing method with capillary electrophoresis. A total of 47 mutations of *p53* were found, in 45 of 131 cases (34%). Mutations were statistically associated with lymphatic invasion (p=0.03) and lymph node metastasis (p=0.02). Kaplan-Meier survival curves for the patients with *p53* mutations were likely to exhibit shortened survivals, but the difference was not statistically significant (p=0.078). In our evaluation of each exon in relationship to survival, *p53* mutations in exon 7 correlated significantly with poor prognosis (p=0.041). In multivariate analysis, *p53* mutation emerged as an independent marker for prognostic hazard ratio=1.650 (p=0.015). However, exon 7 mutations were not related to survival, as well other exons and specific type of mutations. Investigation of *p53* mutation overall was considered to be a clinically usefull approach for determining the prognosis of patients with colorectal cancer.

Hirosaki Med. J. 56 : 69—78, 2005

Key words: colorectal cancer; *p53* mutation; direct sequencing method.

原 著

直接シークエンス法による大腸癌手術症例における *p53*遺伝子変異の予後の検討

渡 邉 智 子　田 村 好 弘　対 馬 健 一　棟 方 昭 博

抄録 大腸癌手術131例について，*p53*遺伝子変異の臨床病理学的因子と予後との関連を検討した．キャピラリー電気泳動による直接シークエンス法を用いて，*p53*遺伝子のexon 5-9の変異を検出した．大腸癌131症例中45例 (34%) に47個の変異を認めた．臨床病理学的因子では，リンパ管侵襲陽性群 (p=0.03) とリンパ節転移陽性群 (p=0.02) において有意に変異がみられた．Kaplan-Meier法で*p53*変異陽性群は予後不良の傾向がみられたが，有意差はなかった (p=0.078)．exon別では，exon 7は野生型と比し有意に予後不良であった (p=0.041)．多変量解析で*p53*変異陽性群は相対危険度が1.650で，予後不良因子であった (p=0.015)．exon 7は，別のexonやある特定部位の変異と同様に有意差が認められなかった．大腸癌において*p53*遺伝子変異の検討は予後予測に有用であると考えられた．

弘前医学 56 : 69—78, 2005

キーワード: 大腸癌; *p53*遺伝子変異; 直接シークエンス法.

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Received for publication, December 16, 2004
Accepted for publication, February 14, 2005

弘前大学医学部内科学第一講座
別刷請求先：渡邉智子
平成16年12月16日受付
平成17年 2 月14日受理

Introduction

With the rapid progress in molecular biology in recent years, the mechanisms of carcinogenesis in cancers of the digestive tract have been further elucidated. It is widely accepted that multiple genetic errors affecting protooncogenes, such as ras, and tumor suppressor genes, such as *APC*, *p53* and *DCC*, are involved in the development and /or progression of colorectal cancer. Intensive screening for genetic alteration led to the identification of two types of colorectal cancer that are distinguished by their carcinogenesis processes. The first group, named LOH (loss of heterozygosity)-positive, is characterized by hyperploidy and allelic losses involving preferentially chromosome 18q and chromosome 17p. The second group, called multiple microsatellite loci (MSI)-positive cancer, is characterized by genetic instability at microsatellite loci. Although colorectal cancer cells are characterized by specific microsatellite alterations, the same signaling pathways, WNT/Wingless, K-ras, transforming growth factor (TGF) β and *p53* pathway, could be implicated in tumor progression[1].

In colorectal cancer, mutations of the *p53* genes are found frequently, and appear to play an important role in the development and progression. The wild type *p53* protein produced by the normal gene is activated by DNA damage. G1 phase arrest is induced in these cells and the damaged DNA is repaired during this period. In cases in which it is not possible to repair the damage, genes such as BAX serve to induce apoptosis, thus inhibiting the survival of cells with irreparably damaged DNA. However, when the *p53* gene is damaged by point mutations, for instance, its function is abolished which may lead to the development of cancer[2].

The *p53* gene consists of 11 exons encoding a protein that comprises a transactivation domain, a core domain/sequence-specific DNA-binding domain and a COOH-terminal domain. The great majority of the mutations are clustered in the core domain (120-292bp). This domain is important for DNA-specific binding and essential for *p53* function. In this domain, there are conserved regions (region II. codons 112-141; region III. codons 171-181; region IV. codons 234-258; region V. codons 271-286) where the amino acid sequence is conserved even among different animal species[3]. Furthermore, there are important structural motifs that contain the L2 loop (codons 163-195), the L3 loop (codons 236-251) and the LSH motif (codons 276-286) in the same domain[4]. Most *p53* mutations are missense mutations that affect different structural motifs, therefore, different prognostic significance. Several reports have indicated that specific *p53* mutations are associated significantly poor prognosis[5-10], however the results remain controversial.

In the present study, we prospectively examined tumor specimens from 131 patients with colorectal cancer for mutations of the *p53* gene using the direct sequencing method and analyzed on prognosis to evaluate different types of mutations as well as *p53* mutations.

Materials

The study group consisted of 131 consecutive patients with colorectal cancer who underwent surgical resections at Hirosaki University Hospital from July 1994 to July 1999. The mean age at diagnosis was 64.2 years old. We excluded cases of ulcerative colitis, hereditary non-polyposis colorectal cancer and familial colorectal adenomas from this study. After removing

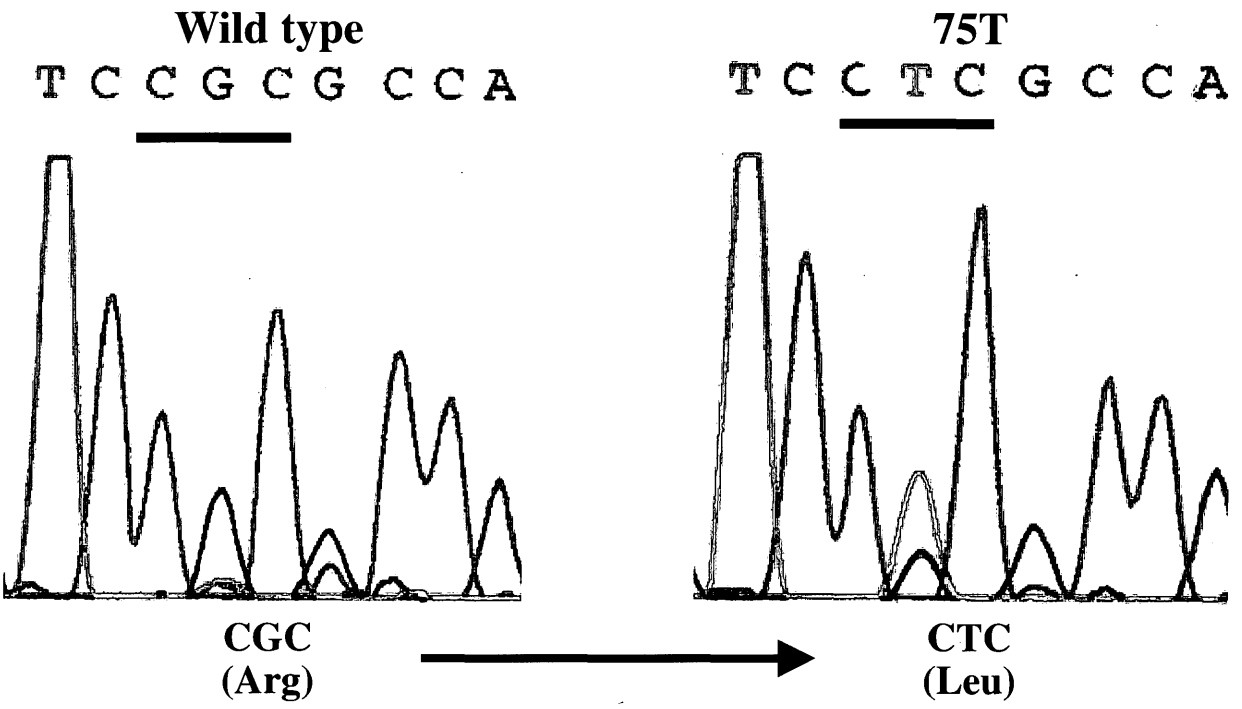

Figure 1 Sequencing of exon 5 shows a G to T point mutation at codon 158 in 75T sample.

these exclusions, the study group included 82 males and 49 females ranging from 29 to 83 years old. Median follow-up period was 46 months (ranging 14-94 months). The Ethics Committee of Hirosaki University School of Medicine approved this study protocol, and all patients provided written informed consent.

Methods

Extraction of genomic DNA

From each surgical specimen, a sample of the tumor was resected, frozen and stored at 80℃ until further use. The samples were later digested by proteinase K and genomic DNA was extracted with phenol-chloroform.

Amplification with the PCR method

To the primers of 40 μM, 200 μM of dNTP, 1.5 mM of $MgCl_2$, 0.5 units of Taq polymerase (Perkin-Elmer, NJ) and 0.1 μg of genomic DNA were added to yield a total volume of 50 μl. After mineral oil was added, exons 5-9 of *p53* were amplified with PCR method. Each PCR was carried out for 35 cycles. The conditions of the reaction were as follows: denaturation at 94℃ for 1 minute, annealing at 55℃ for 1 minute and extension at 72℃ for 1 minute. The primers used were: (5'-TG TTCACTTGTGCCCTGACT-3' for the sense strand, 5'-GAGCAATCAGTGAGGAATCA G-3' for the anti-sense strand in exon 5), (5'-T GGTTGCCCAGGGTCCCCAG-3', 5'-CGGAGG GCCACTGACAACCA-3' in exon 6), (5'-CTTG CCACAGGTCTCCCCAA-3', 5'-AGGGGTCAG CGGCAAGCAGA-3' in exon 7), (5'-TTGGGAG TAGATGGAGCCTG-3', 5'-GAGTGTTAGACT GGAAACTTT-3' in exons 8 and 9).

Sequencing

Direct sequencing was performed using a Dye Terminator Cycle Sequencing FS Ready Kit (Perkin-Elmer, NJ). Then, 8 μl of Terminator Premix, 1 μl of PCR product, 3.2 pmol of sequencing primer, water and DNA were added to yield a total volume of 20 μl. Each PCR was carried out for 25 cycles under the following conditions: denaturation at 96℃ for 30 seconds, annealing at 50℃ for 15

seconds and extension at 60℃ for 4 minutes. The primers used were (5'-TTCAACTCTGT CTCCTTCCT-3', 5'-CAGCCCTGTCGTCTCTC CAG-3' in exon 5), (5'-GCCTCTGATTCCTCA CTGAT-3', 5'-AGTTGCAAACCAGACCTCAG -3' in exon 6), (5'-GTGTTATCTCCTAGGTT GGC-3', 5'-TGTGCAGGGTGGCAAGTGGC-3' in exon 7), (5'-TATCCTGAGTAGTGGTAAT C-3', 5'-TAAGAGGTCCCAAGACTTAG-3' in exons 8 and 9).

Using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, CA), capillary electrophoresis was performed and the total base sequence of the introns adjacent to each exon was determined. Sequencing was performed for all tumors on both the sense and anti-sense strands (Figure 1).

Statistical analysis

To analyze the correlations between various clinicopathological factors and *p53* gene mutations, the chi square test was used. Survival curves were prepared with the Kaplan-Meier method, and the log rank test was employed. Associations between

Table 1 Mutations of the *p53* gene in 131 patients with colorectal cancer

	Exon	Codon	Nucleotide		change	Amino	Acid	change
1	5	151	CCC	→	CAC	Pro	→	His
2	5	158	CGC	→	CTC	Arg	→	Leu
3	5	159	GCC	→	CCC	Ala	→	Pro
4	5	161	GCC	→	GAC	Ala	→	Asp
5	5	173	GTG	→	TTG	Val	→	Leu
6	5	175	CGC	→	CAC	Arg	→	His
7	5	175	CGC	→	CAC	Arg	→	His
8	5	175	CGC	→	CAC	Arg	→	His
9	5	175	CGC	→	CAC	Arg	→	His
10	5	175	CGC	→	CAC	Arg	→	His
11	5	175	CGC	→	CAC	Arg	→	His
12	5	175	CGC	→	CAC	Arg	→	His
13	5	180	GAG	→	TAG	Glu	→	stop
14	6 intronic	donor site	cctag	→	cctgg			
15	6	192	CAC	→	TAC	His	→	Tyr
16	6	195	ATC	→	ACC	Ile	→	Thr
17	6	195	ATC	→	AGC	Ile	→	Ser
18	6	195	ATC	→	AGC	Ile	→	Ser
19	6	196	CGA	→	TGA	Arg	→	stop
20	6	196	CGA	→	TGA	Arg	→	stop
21	6	196	CGA	→	TGA	Arg	→	stop
22	6	204	GAG	→	TAG	Glu	→	stop
23	6	213	CGA	→	TGA	Arg	→	stop
24	6	213	CGA	→	TGA	Arg	→	stop
25	6	213	CGA	→	TGA	Arg	→	stop
26	6	213	CGA	→	TGA	Arg	→	stop
27	6	214	CAT	→	CGT	His	→	Arg
28	6	215	AGT	→	ATT	Ser	→	Ile
29	7	244	GGC	→	GCC	Gly	→	Ala
30	7	244	GGC	→	TGC	Gly	→	Cys
31	7	245	GGC	→	AGC	Gly	→	Ser
32	7	248	CGG	→	TGG	Arg	→	Trp
33	7	248	CGG	→	TGG	Arg	→	Trp
34	7	248	CGG	→	TGG	Arg	→	Trp
35	7	248	CGG	→	CAG	Arg	→	Gln
36	7	248	CGG	→	CAG	Arg	→	Gln
37	7	248	CGG	→	CAG	Arg	→	Gln
38-1	7	253	ACC	→	GCC	Thr	→	Ala
-1	7	258	GAA	→	GAC	Glu	→	Asp
-2	7 intronic	donor site	cctag	→	cctgg			
39	8	273	CGT	→	CAT	Arg	→	His
40	8	273	CGT	→	CAT	Arg	→	His
41	8	282	CGG	→	TGG	Arg	→	Trp
42	8	282	CGG	→	TGG	Arg	→	Trp
43	8	282	CGG	→	TGG	Arg	→	Trp
44	8	284	ACA	→	AAA	Thr	→	Lys
45	8	306	CGA	→	TGA	Arg	→	stop

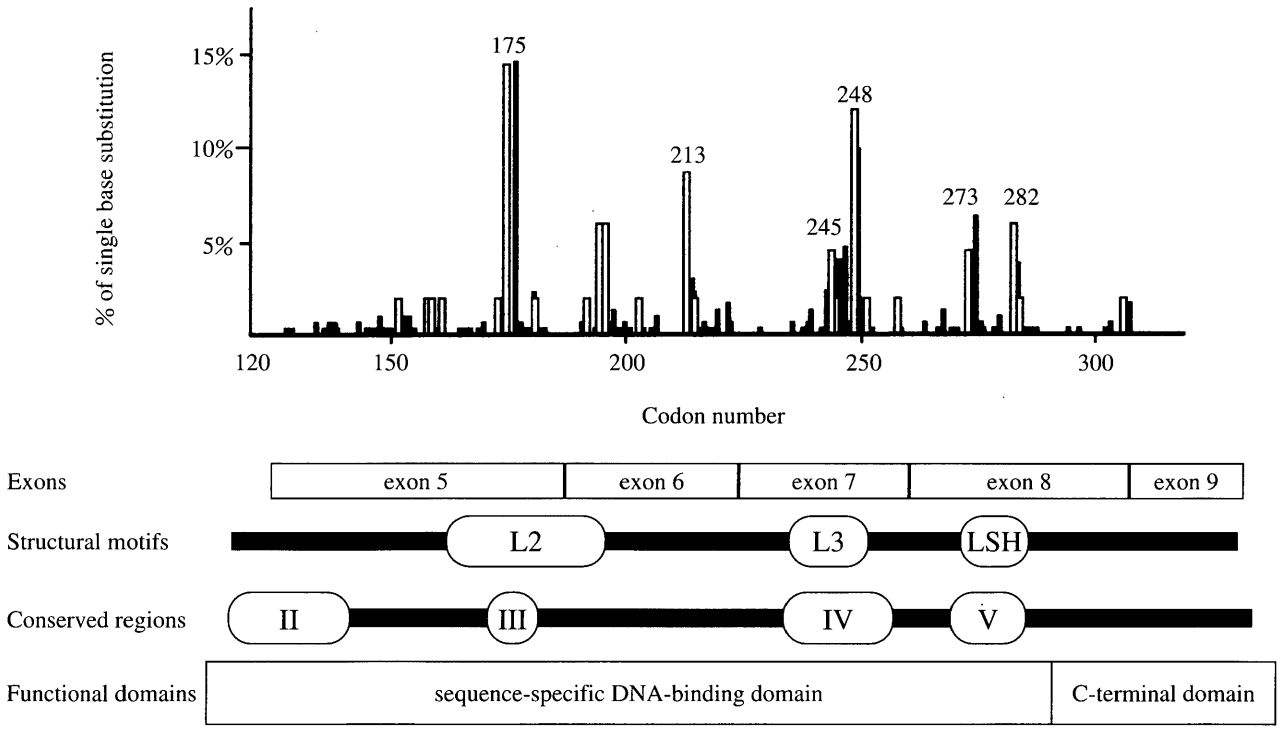

Figure 2 Comparison of distribution of *p53* mutations between International Agency for Research on Cancer (IARC) database (filled columns) and the present study (blank colomns). (IARC URL=http://www.-p53.iarc.fr/index.html)

survival and *p53* mutations also were determined using Cox's proportional hazard models. A P value of less than 0.05 was considered statistically significant.

Results

A total of 47 mutations were discovered among 45 of the 131 tumors (34%) (Table 1). One case involved synchronous tumors, one of which demonstrated 2 different mutations, while the other had a single point mutation at the intronic donor site. All 47 mutations were point mutations. There were 34 transitions constituting base changes between purines or pyrimidines, and 13 transversions involving mutations from purine to pyrimidine or pyrimidine to purine. Neither base deletions nor insertions were identified.

Specifically, the 47 point mutations were as follows: 35 missense mutations (74%) directing the cell to synthesize a mutant protein, 10 nonsense mutations (22%), signaling a stop codon to terminate polypeptide strand and 2 point mutations (4%) at intronic donor sites. There were 13 mutations (28%) at exon 5, 14 (30%) at exon 6, 11 (23%) at exon 7, 7 (15%) at exon 8 and 2 (4%) at the intronic donor site. In the conserved region, there were 25 mutations (53%) of *p53*, representing more than one half of all mutations identified. We compared with the distribution of *p53* mutation of the International Agency for Research on Cancer (IARC) database in colorectal cancer (Figure 2). Apparently the higher proportion of codon 213 mutations was found in the present study, but it was not significant. Exception of codon 213, the distribution was similar to IARC database.

Correlation of *p53* mutations with clinical and pathological factors

Evaluations were performed to identify any relationships between each of the clinical and pathologic factors and the *p53* gene mutations (Table 2). Of the 103 specimens demonstrating lymphatic invasion, *p53* mutations were found in 41 (40%). This percentage was significantly higher than the 5 mutations of *p53* (17%) occurring in the 29 cases without lymphatic invasion (p=0.03). The 29 cases of *p53* mutations (46%) identified in the 62 patients with lymph node metastases represented a significantly higher number than the 16 mutations of *p53* (23%) seen in the 69 patients without lymph node spread (p=0.02). Tumors of advanced TNM stage showed significantly higher mutation rates (p=0.03). There were no significant correlations between *p53* mutations and age, sex, tumor site, histological classification or venous invasion. Furthermore, we performed the statistical analysis of the each exons, the result was that exon 7 mutations were significantly more frequent in higher stage (p=0.01) (data not shown). No relationships were seen between exon 7 mutations and other factors.

Table 2 Evaluation of *p53* mutations and clinicopathological factors

	Wild type n=86 (65.7%)	Mutation n=45 (34.3%)	χ^2	P-value
Age				
<50	10	8 (44.4%)		
≦50 and <65	27	11 (28.9%)	2.02	0.57
≦65 and <75	38	17 (30.9%)		
≦75	11	9 (45.0%)		
Sex				
Male	51	31 (37.8%)	1.16	0.28
Female	35	14 (28.6%)		
Location				
Rectum	38	20 (34.4%)		
Left colon	17	15 (46.8%)	3.43	0.18
Right colom	31	11 (26.1%)		
Gross appearance				
0	8	0 (0%)		
1	5	3 (37.5%)		
2	52	30 (38.6%)	5.03	0.41
3	17	11 (39.2%)		
4	1	0 (0%)		
5	3	2 (40.0%)		
CEA				
<10	68	31 (31.3%)		
≦10 and <50	12	8 (40.0%)	1.99	0.40
≦50	6	3 (50.0%)		
Histology				
Well-differentiated	33	15 (31.2%)		
Moderately-differentiated	39	27 (40.9%)	4.08	0.25
Poorly-differentiated	5	3 (37.5%)		
Mucinous	9	1 (10.0%)		
TMN stage				
1	19	5 (20.8%)		
2	32	11 (25.6%)	8.23	0.04
3	24	19 (44.2%)		
4	11	10 (47.6%)		
Venous invasion				
Negative	45	23 (33.8%)		
Positive	41	23 (35.9%)	0.01	0.91
Lymphatic duct invasion				
Negative	24	5 (17.2%)		
Positive	62	41 (39.8%)	4.79	0.03
Lymph node metastasis				
Negative	53	16 (23.1%)		
Positive	33	29 (46.7%)	4.80	0.02

p53 gene mutation and prognosis

Patients with *p53* mutations were likely to have shorter survivals than those without such mutation, although this difference was not statistically significant (Figure 3A). When we performed univariate survival analysis of the function of the affected exons, patients with exon 7 mutations in particular demonstrated the shortest survival among those with any mutations (p=0.041) (Figure 3B).

Multivariate analysis was performed to assess the relative influence of the following factors: age, sex, histology, TNM stage and tumor site. TNM stage was found to be an independent prognostic indicator of shorter survival (p=0.001) (Table 3). And *p53* mutations were a significantly poor prognostic indicator (p=0.015). On the other hands, with adjusting for the same factors, we found no significantly statistical differences among patients who had tumors with exon

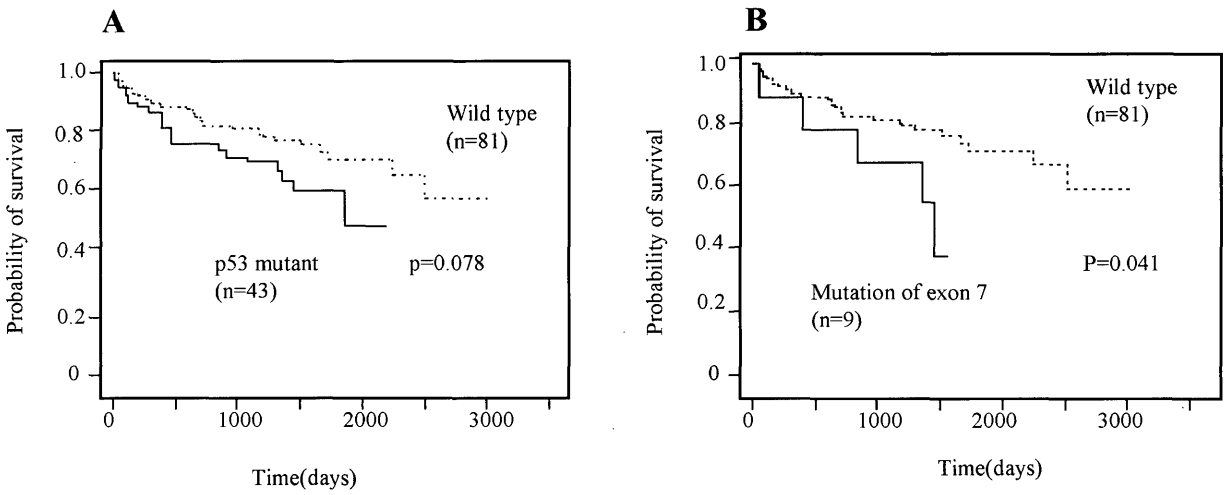

Figure 3 Survival curves for colorectal cancer patients.
A, patients with *p53* mutations and those with wild type *p53*.
B, patients with exon 7 mutations and those with wild type *p53*.

Table 3 Multivariate analysis of prognostic factors affecting overall survival of patients with colorectal cancer

Variable	Category	Hazard ratio	95% CI [a]	P-value
Age	Continuous	1.008	0.991-1.025	0.370
Sex	Female vs. Male	1.352	0.909-2.011	0.136
Stage	Continuous	1.398	1.149-1.702	0.001
Differentiation	poorly, mucinous vs. well, moderate	1.249	0.719-2.171	0.430
Location	rectum, left colon vs. right colon	1.267	0.838-1.915	0.261
p53 status	Mutation vs. Wild type	1.650	1.101-2.472	0.015
Exon 5	Mutation vs. Wild type	1.376	0.380-4.980	0.627
Exon 6	Mutation vs. Wild type	1.230	0.401-3.768	0.718
Exon 7	Mutation vs. Wild type	1.518	0.510-4.515	0.453
Exon 8	Mutation vs. Wild type	1.523	0.343-6.763	0.580
Conserved region	Mutation vs. Wild type	1.436	0.628-3.285	0.391
Missesnse	Mutation vs. Wild type	1.508	0.695-3.29	0.298
Nonsense	Mutation vs. Wild type	0.595	0.214-1.650	0.318
Hot spots (codon175,245,248,273,282)	Mutation vs. Wild type	1.569	0.646-3.811	0.320
Structural motifs (L2 loop, L3 loop, LSH domain)	Mutation vs. Wild type	1.139	0.412-3.147	0.802

[a] confidence interval.

7 mutations, since exon 7 mutations were prevalent in higher stage. As well, other exon 5, 6 and 8 mutations were not significant poor prognosis. And concerning other types of *p53* mutation, which were missense mutation, nonsense mutations, conserved regions, hot spots (codon 175, 245, 258, 273, 282) and structural motifs (L2 loop, L3 loop, LSH domain), there were no significant differences.

Discussion

In our screening for mutations in exons 5-9 of *p53* from colorectal cancer of 131 cases, we observed a mutation frequency of 34%. Previous studies have reported a fairly wide range of values (28-63%)[5-19]. Some of these previous studies[10-12] were relatively small, with less than 100 patients. The observed frequency of *p53* mutations in our population was higher than that (28%) reported by Elsaleh et al.[13], but was very similar to that (36%) found by Soong et al.[14] and Tang et al.[15]. This variability can be explained by the different methods used to assess *p53* mutations (direct sequencing method, denaturing gradient gel electrophoresis, temperature gradient gel electrophoresis and SSCP). And the difference in tumor etiology among the populations examined may have led to the difference in frequency. In other words, racial composition differences between our study group and that of previously studied groups may partially explain apparent etiologic discrepancies. In addition or even alternatively, it is possible that contamination of tumor DNA in connective tissue-rich tumors by normal cellular DNA caused the relatively low frequency.

It has been stated that the mutational status of the *p53* gene is not associated with the histological grade or stage of disease[9,11,16]. To the contrary, some studies stated that *p53* mutations were significantly associated with TNM stage and lymph node metastasis[6,17]. In our study, we observed that *p53* mutations were significantly related to stage, and lymphatic invasion and lymph node metastasis. This would account for the high frequency of *p53* mutations among patients with advanced stage disease and suggests that the role of *p53* mutations may relate to the factors involved in tumor progression.

The relationship between *p53* mutations and prognosis has been evaluated in many previous studies, with some showing no significant relationship with prognosis[13,16,18], some showing an association with poor prognosis[5-12,17] and some even showing a relationship to improved prognosis[14,19,20]. In univariate analysis we found that survival of patients with *p53* mutations was poorer than that of patients with wild type *p53*. However, in multivariate analysis, it appeared that *p53* mutation was an independent prognostic factor when adjusted for age, sex, histology, TNM stage and tumor site. Our study essentially supports the previous studies in which *p53* was associated with poor survival. The mechanism by which mutations of *p53* are associated with poorer survival is as yet unclear. Mutated *p53* proteins have lost their function in cell cycle arrest, apoptosis, inhibition of tumor growth and preservation of genetic stability. Thus, an aggressive tumor with selective growth advantage, accumulating additional genetic alterations as well as conferring resistance to radio- or chemotherapy, may be the result of *p53* mutation[12].

It has been observed that all point mutations are not functionally equivalent, and the data generated by DNA sequencing method support this. Previous studies have also suggested that specific classes of *p53* mutations have a particularly poor prognosis.

Goh et al.[5] found that mutations in the hot spot of codon 175 were associated with poor prognosis. Samowitz et al.[6] who studied a large population noted that mutations in the 245 hot spot were significantly prognostic impacts. Jernvall et al.[10] and Goh et al.[5] reported that patients with point mutations in the conserved regions of the *p53* gene had significantly worse outcomes than those with base changes outside of these regions. Borrensen-Dale et al.[7] and Russo et al.[8] showed that patients with mutations in the L3 domain had the worst survival compared to all other patients. Iniesta et al.[9] found that patients with exon 7 mutations had a poor prognosis. In this study, univariate analysis showed that the survival of patients who had exon 7 mutation was characterized by significantly poor prognosis, as shown in previous study. Exon 7 of *p53* encodes for amino acids 225-261 in the protein, which is part of the core domain (residues 102-292) that is essential for sequence-specific DNA binding. The face of *p53* that interacts with DNA contains the majority of mutational hot spots associated with human cancer. This remarkable discovery has allowed Cho et al.[3] to explain the molecular basis for p53 inactivation. They identified two major classes of *p53* mutants. The first involves residues that interact with DNA. Missense mutations at these sites (for instance, Arg248 and Arg273) inactivate *p53* by eliminating crucial DNA contacts. The second class of mutant exhibits an abnormal structure owing to missense mutation at sites important for the conformational architecture of the core domain. Such mutations (for instance, Arg175, Gly245, Arg249 and Arg282) destabilize the *p53* tertiary structure, resulting in a loss of sequence-specific DNA binding capacity. We found that exon 7 mutations of *p53* were prevalent in stage III and IV. Therefore, in multivariate analyses, exon 7 mutations appeared to be an insignificant prognostic factor as well other exon 5, 6 and 8 mutations. And we evaluated the prognostic significance of various types of *p53* mutation. In this study we found no significant prognostic value for any of the different types of mutations. Because of relatively small number of patients with *p53* mutation, we were unable to determine the influence of mutation type on survival.

In summary, *p53* mutations in colorectal cancer were significantly more common in tumors of advanced stage, tumors with lymphatic duct invasion, and seemed to be in the process of tumor progression. *p53* mutations were a useful prognostic factor and the patients with these mutations require more careful follow-up than those without mutations. However, specific classes of mutations could not to be an independent predictor of poor prognosis.

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