Pergamon

European Journal of Cancer Vol. 33, No. 7, pp. 1136–1140, 1997

Printed in Great Britain
0959-8049/97 $17.00 + 0.00

PII: S0959-8049(97)00080-4

Original Paper

TP53 Mutations in Stage I Gallbladder Carcinoma with Special Attention to Growth Patterns

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32 stage I cases of gallbladder carcinoma (GC) were examined to evaluate *TP53* mutations with special attention to growth patterns. Their growth patterns were classified into two types: polypoid (P-type) and flat (F-type). 16 cases of GC were classified as P-type and 16 as F-type. p53 immunohistochemistry was performed using a mouse monoclonal anti-p53 antibody. Mutations in exons 5–8 were examined by polymerase chain reaction single strand conformation polymorphism (PCR-SSCP) and direct sequencing. The incidence of p53 immunoreactivity was greater in the cases of F-type (11/16, 69%) than those in P-type (14/16, 25%) ($P < 0.05$). PCR-SSCP or direct sequencing revealed that *TP53* mutations were detected in all cases positive for p53 protein. These results suggest that *TP53* mutations may contribute to the carcinogenesis of the F-type GC, and that this pathway in the F-type may differ from that in the P-type GC. © **1997 Elsevier Science Ltd.**

Key words: gallbladder carcinoma, *TP53*, growth pattern, stage I, carcinogenesis

Eur J Cancer, Vol. 33, No. 7, pp. 1136–1140, 1997

INTRODUCTION

GALLBLADDER CARCINOMA is a relatively uncommon disease accounting for approximately 0.3–0.7% of all cancers [1]. It is more prevalent in Japan than in the Western world [2]. In Japan, gallbladder carcinomas are classified according to tumour depth using the criteria of the Japanese Society of Biliary Surgery [3]. According to this criteria, mucosal (m) or proper muscle (pm) tumours are classified as "early gallbladder carcinomas" [3]. Previous studies of early gallbladder carcinomas have revealed an incidence of "flat type" tumours of 74%, and that of "polypoid type" tumours of 26% [4].

Recent advances in molecular biology revealed that several genetic alterations are involved in the carcinogenesis of various malignancies including colorectal carcinomas [5, 6] and gallbladder carcinomas [7–10]. Vogelstein and associates have suggested that *RAS* gene mutations are early events in colorectal carcinogenesis in a genetic model based on the typical adenoma–carcinoma sequence through polypoid adenomas [11]. Yamagata and associates reported that the incidence of *K-RAS* mutations in non-polypoid colorectal carcinomas was lower than that in polypoid colorectal carcinomas, and have suggested that the pathway of non-polypoid colorectal carcinomas might differ from the genetic model previously reported by Vogelstein and associates [11, 12]. In gallbladder carcinomas, metaplastic changes are frequently found in non-cancerous regions adjacent to the cancer regions, and a sequence of metaplastic changes to carcinoma has been suggested [13, 14]. Recent studies have indicated that mutations of *TP53*, a tumour suppressor gene, may play an important role in the carcinogenesis of gallbladder carcinoma [8–10, 15, 16]. However, there have been no previous reports on *TP53* mutations in gallbladder carcinomas with special attention to growth patterns. The present study was designed to determine the relationship between *TP53* mutations and the growth patterns of stage I gallbladder carcinomas.

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Received 26 Jul. 1996; revised 13 Dec. 1996; accepted 2 Jan. 1997.

MATERIALS AND METHODS

Tumour material

32 cases of gallbladder carcinoma (GC) were examined, which were surgically resected at Hiroshima University Hospital and its affiliated hospitals from 1985 to 1995. Informed consent was obtained from all patients. All cases

Table 1. *TP53 mutations in flat type GC*

No.	Age	Sex	Histology	Depth	Immunohistochemical study	Exon (SSCP)	Codon	Nucleotide substitution	Amino acid change
1	77	F	well	pT1a	+	5	147	GTT > GGT	Val > Gly
						8	277	TGT > TGC	Cys > Cys
2	71	F	well	pT1a	+	5		NE	
3	61	F	well	pT1a	+	5		NE	
4	70	M	well	pT1a	+	6		NE	
5	58	F	well	pT1a	+	7	249	AAG > AAC	Lys > Asn
6	78	F	well	pT1a	+	7	246	ATG > TTG	Met > Leu
7	74	F	well	pT1a	+	7	249	AAG > AAC	Lys > Asn
8	85	F	well	pT1a	+	8	270	TTT > TTG	Phe > Leu
9	69	F	well	pT1a	+	8		NE	
10	73	F	well	pT1b	+	5	147	GTT > GGT	Val > Gly
11	55	F	well	pT1b	+	6	202	CGT > CAT	Arg > His

The tumour classification is according to the TNM staging system [17]. GC, gallbladder carcinomas; SSCP, single-strand conformation polymorphism; M, male; F, female; well, well-differentiated adenocarcinoma; pap, papillary adenocarcinoma; pT1a, tumour invades the mucosa; pT1b, tumour invades the muscle layer; NE, not examined.

were diagnosed by histological findings, and were classified as stage I according to the TNM staging system [17]. In addition, all cases were classified macroscopically into one of two types according to the General Rules for the Gastric Cancer Study in Surgery and Pathology [18]. The polypoid type (P-type) consisted of type I (protruded) tumours, and the flat type (F-type) consisted of type IIa (elevated) or type IIb (flat) tumours. There were 16 P-type cases of GC and 16 F-type cases of GC. For the P-type, there were 9 papillary adenocarcinomas and 7 well-differentiated adenocarcinomas. For the F-type, all cases were well-differentiated adenocarcinomas. There were 8 men and 24 women, ranging in age from 41 years to 86 years (mean age: 66). All resected tissues were fixed in 10% buffered formalin and embedded in paraffin. Sections of the specimens, 4 μm in thickness, were cut.

We also used four pancreatic cancer cell lines, MIAPaCa-2 (supplied from the Japanese Cancer Research Resource Bank), BxPC3, Capan-1 and PANC-1 (purchased from the American Type Culture Collection). They were used as positive controls for mutations in exons 5–8 of *TP53* [19, 20].

p53 immunohistochemistry

The avidin–biotin complex method was used for p53 immunostaining. The section was immunostained for p53 protein after microwave treatment at 90°C for 4 min. A mouse monoclonal anti-p53 antibody (DO-7) was purchased from Dako Japan Co. Ltd., Kyoto. The reaction was performed at a dilution of 1:20 at 4°C overnight. When more than 10% of tumour nuclei were stained for p53, the section was judged as positive for p53 protein.

DNA preparation

DNA was extracted from the cases positive for p53 by immunohistochemistry. One section from each paraffin-embedded tissue was stained with haematoxylin and eosin, and the tumour cell areas were removed from the serial 4 μm sections with a 27-G syringe needle under a stereoscopic microscope. Each sample from the paraffin-embedded tissues and four pancreatic cancer cell lines were incubated in 100 μl of lysis buffer (10 μM Tris-HCl, 50 mM KCl, 2.5 mM $MgCl_2$ and 0.45% Tween-20) containing proteinase K (1 mg/ml) at 40°C for 12 h. After inactivating the proteinase K by incubating at 100°C for 10 min, the samples were used for polymerase chain reaction (PCR) analysis.

Detection of TP53 *mutations*

Polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP). The amplifications were performed using genomic DNA extracted from the samples as described above. Exons 5–8 of the *TP53* gene were amplified separately by PCR, and the mutations were screened by SSCP as previously reported [21]. The PCR amplifications were performed in 5 μl reaction mixtures containing 50 ng of DNA, [α-^{32}P]CTP (Amersham Japan Co. Ltd., Tokyo), 0.2 μM of the proper pair of each primer (Takara Shuzo Co. Ltd., Kyoto), 15 μM of each deoxyribonucleoside triphosphate, PCR buffer and Taq polymerase (Perkin-Elmer Cetus, Norwalk, Connecticut, U.S.A.). The mixtures were subjected to 35 cycles of PCR amplification (each cycle consisted of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min). The amplified samples were then heated at 95°C for 5 min, and loaded on to 5% polyacrylamide gel containing 5% glycerol. After electrophoresis at room temperature, the gel was exposed to X-ray film for 10–12 h. The PCR amplification was performed at least twice for each sample, and only the reproducible cases were

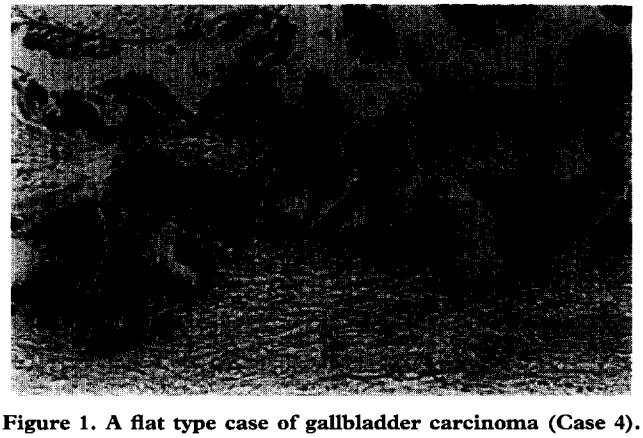

Figure 1. A flat type case of gallbladder carcinoma (Case 4). Well-differentiated adenocarcinoma cells invading the mucosa and positive for p53 are visible (×100).

Table 2. TP53 *mutations in polypoid type GC*

No.	Age	Sex	Histology	Depth	Immunohistochemical study	Exon (SSCP)	codon	Nucleotide substitution	Amino acid change
13	41	M	pap	pT1a	+	5	137	CTG > CTT	Leu > Phe
						5	145	CTG > CTC	Leu > Leu
						5	146	TGG > CGG	Trp > Arg
14	74	F	pap	pT1a	+	5	190	CCT > ACT	Pro > Thr
15	75	M	well	pT1a	+	8	270	TTT > TTG	Phe > Leu
16	59	M	well	pT1a	+	8	268	GAA > TTA	Glu > Leu

The tumour classification is according to the TNM staging system [17]. See footnotes in Table 1.

used. The synthetic oligonucleotides used as primers have been described previously [9].

Direct sequence. The amplified PCR products were separated by electrophoresis using low melting point agarose, and purified using Wizard PCR Prep (Promega, Madison, Wisconsin, U.S.A.). Identification of mutations in *TP53* was done by direct sequencing of the DNAs, using a Circum Vent™ Thermal Cycle Dideoxy DNA Sequencing Kit (Biolabs, Nebraska, U.S.A.). The primers of exons 5 and 6 were the same as described in the method of PCR-SSCP. The primers of exons 7 and 8 were as follows:

Exon 7, sense: 5'CTTGCCACAGGTCTCCCCAA3' and antisense: 5'AGGGGTCAGCGGCAAGCAGA3'.

Exon 8, sense: 5'TTGGGAGTAGATGGAGCCTG3' and antisense: 5'CTGCTTGCTTACCTCGCTTA3'.

Statistical analysis. Statistical analysis was performed using Fisher's Exact Test. A P value < 0.05 was considered statistically significant.

RESULTS

p53 immunohistochemistry

In the cases of F-type, 11 out of 16 (69%) were positive for p53 (Table 1 and Figure 1). In the cases of P-type, 4 out of 16 (25%) were positive for p53 (Table 2 and Figure 2). The incidence of p53 immunopositivity in the cases of F-type was significantly greater than that in the case of P-type ($P < 0.05$).

Detection of TP53 *mutations*

In the cases of F-type, SSCP analysis detected *TP53* mutations (exons 5, 6, 7 and 8) in all 11 cases positive for p53 protein. Direct sequencing could detect point mutations in exons 5, 6, 7 and 8 in 7 cases (Table 1).

In the cases of P-type, SSCP analysis detected *TP53* mutations (exons 5 and 8) in all 4 cases positive for p53 protein (Figure 3). Direct sequencing could detect point mutations in exons 5 and 8 in the same cases (Table 2 and Figure 4). *TP53* mutations were detected in 2 out of 9 (22%) papillary adenocarcinomas, and in 2 out of 7 (29%) well-differentiated adenocarcinomas.

Regardless of growth patterns, in all cases negative for p53 protein, no mutations were detected by PCR-SSCP in exons 5–8 (data not shown).

Figure 2. A polypoid type case of gallbladder carcinoma (Case 13). Papillary adenocarcinoma cells invading the mucosa and positive for p53 are visible (×40).

DISCUSSION

In stage I GC, it has been reported that the incidence of "flat type" tumours is greater than that of "polypoid type" tumours [4]. In GC, a sequence of metaplastic changes to carcinoma has been suggested [12–14]. However, it is unclear whether carcinogenesis of the polypoid type GC differs from that in flat type GC.

There have been few previous studies on *K-RAS* mutations in colorectal carcinoma with special attention to growth patterns. The investigators in these studies reported that the incidence of *K-RAS* codon 12 mutations in flat or elevated cancers is significantly lower than that in polyp-forming cancers [11, 22]. These reports also suggested that *K-RAS* gene mutations may correlate with morphology or the clinical features in flat or elevated cancers, and that these cancers may originate via a pathway differing from the adenoma–carcinoma sequence [11, 22].

There have also been few previous studies that have examined *TP53* mutations in colorectal carcinoma with special attention to growth patterns. It has been reported that there is no significant difference in the frequency of *TP53* mutations between flat- and polypoid type colorectal

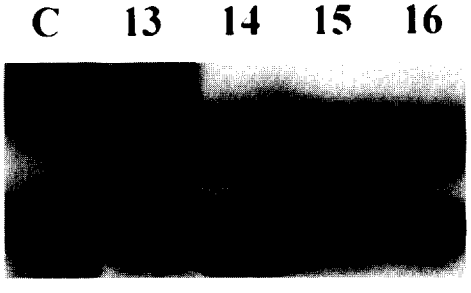

Figure 3. PCR-SSCP analysis in exon 5 of *TP53* in the polypoid type cases of gallbladder carcinoma. Fragments with an abnormal mobility shift were observed in Cases 13 and 14. C: MIAPaCa-2. Numbers refer to the patient numbers in Table 2.

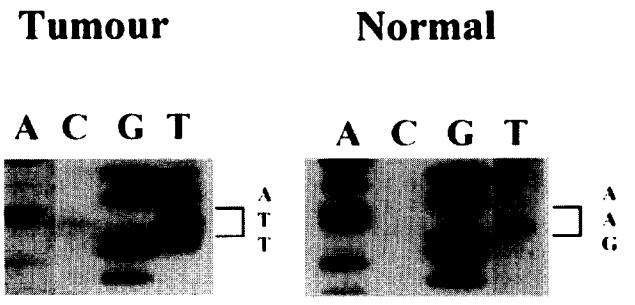

Figure 4. Nucleotide sequence analysis in Case 16. The A → T and G → T transitions in codon 268 are shown.

tumours [23, 24]. The *TP53* tumour suppressor gene protein product plays a pivotal role in normal cell growth and differentiation [25]. *TP53* mutations are associated with cell immortalisation or transformation *in vitro* and development of neoplasm *in vivo*. Recent studies have reported that *TP53* mutations may play important roles in the carcinogenesis of gallbladder carcinoma. The incidence of p53 overexpression detected by immunohistochemistry has ranged from 47% to 92% [8–10, 15, 16], while that of *TP53* mutations detected by PCR-SSCP or direct sequencing has ranged from 31% to 45% [9, 26]. However, there has been no report on *TP53* mutations in the stage I cases of GC with special attention to growth patterns.

Our data demonstrate that the incidence of p53 overexpression in flat type stage I GC (69%) was significantly greater than that in polypoid type stage I GC (25%) ($P < 0.05$). PCR-SSCP or direct sequencing revealed that *TP53* mutations were detected in all cases positive for p53 protein. These results suggest that, if a genetic model based on the typical adenoma–carcinoma sequence through polypoid adenomas was hypothesised, *TP53* mutations may partly contribute to the early stage of carcinogenesis in the polypoid type GC. Furthermore, *TP53* mutations may play an important role in the early stage of carcinogenesis in flat type GC. There have been some reports showing a correlation between p53 and biological features. A significant association between p53 overexpression and the presence of DNA aneuploidy has been reported [27]. In breast cancer, Cervantes and associates have reported that *TP53* gene alterations detected in exons 5–8 correlate significantly with gene amplification, a higher S-phase index and the presence of DNA aneuploidy [28]. In gastric cancer, p53 overexpression is not only a remnant of the mutational inactivation of p53 function, but also promotes tumour growth and lymph node metastasis [29]. From our study, flat type stage I GC demonstrated a high incidence of *TP53* mutations. Therefore, flat type GC that have acquired *TP53* mutations may be aggressive.

In this study, the *TP53* mutational spectrum revealed that the incidence of mutation at an A:T pair in flat type GC, was higher than that in polypoid type GC. T → G transversion was frequently detected in cases of flat type GC. Hollstein and associates reported that a high incidence of p53 mutation at an A:T pair was associated with DNA depurination caused by irritants to mucosa [30]. In this case, T → G transversion detected in the cases of flat type GC could be interpreted as suggestive evidence of the action of some endogenous or exogenous biliary mutagens.

Based on these findings, we conclude that there may be at least two different pathways in gallbladder carcinogenesis with respect to *TP53* mutations and morphology. The first pathway is the polypoid pathway with a low *TP53* mutation frequency, and the second is a non-polypoid pathway with a high *TP53* mutation frequency.

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Acknowledgements—The authors are grateful to Dr M. Yamamoto and Dr F. Shimamoto for histologic interpretations, and to Dr M. Hiraoka for statistical analysis.