

CLINICAL RESEARCH

## Correlation of *p53* gene mutation and expression of P53 protein in cholangiocarcinoma

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### Abstract

**AIM:** To characterize the tumor suppressor gene *p53* mutations and study the correlation of *p53* gene mutation and the expression of P53 protein in cholangiocarcinoma.

**METHODS:** A total of 36 unselected, frozen samples of cholangiocarcinoma were collected. *p53* gene status(exon 5-8) and P53 protein were examined by automated sequencing and immunohistochemical staining, combined with the clinical parameters of patients.

**RESULTS:** *p53* gene mutations were found in 22 of 36 (61.1%) patients. Nineteen of 36 (52.8%) patients were positive for P53 protein expression. There were significant differences in extent of differentiation and invasion between the positive and negative expression of P53 protein. However, there were no significant differences in pathologic parameters between the mutations and non-mutations.

**CONCLUSION:** The alterations of the *p53* gene evaluated by DNA sequence analysis is relatively accurate. Expression of P53 protein could not act as an independent index to estimate the prognosis of cholangiocarcinoma.

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**Key words:** Cholangiocarcinoma; *p53* gene; Mutation; DNA sequence

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### INTRODUCTION

Tumor suppressor gene, besides oncogene, is involved in the development of cancer, which inhibits cell proliferation and formation of tumor. Normally tumor suppressor gene counteracts with oncogene to protect an organism against cancer. The *p53* tumor suppressor gene is the most common mutated gene in human cancer<sup>[1-9]</sup>, occurring in approximately 50% cancers<sup>[10-12]</sup>. Cholangiocarcinoma is among the most common malignant tumors. Mutation of *p53* is one of the most frequently encountered genetic alterations in cholangiocarcinoma. *p53* mutations play a central role in carcinogenesis of cholangiocarcinoma.

Alterations of the *p53* gene can be evaluated by DNA sequencing analysis and immunohistochemistry(IHC). P53 protein overexpression is considered a poor prognosis of cholangiocarcinoma<sup>[13-17]</sup>. But IHC has shown controversial results over that of DNA sequencing analysis. We examined mutation status of *p53* gene by automated sequencing and IHC in 36 cases of cholangiocarcinoma and studied the correlation of *p53* gene mutation with clinical parameters and its clinical significance.

### MATERIALS AND METHODS

#### Patients

A total of 36 unselected, frozen samples were obtained from patients with cholangiocarcinoma who had been treated by surgical resection from April 2000 to May 2005 in the Department of General Surgery of the First Affiliated Hospital of China Medical University and Hepatobiliary Surgery of the Affiliated Yantai Yuhuangding Hospital of Qingdao University Medical College. The types of cholangiocarcinoma included 18 cases of tubular adenocarcinomas, 9 cases of papillary adenocarcinomas, 4 cases of mucoid carcinomas and 5 cases of undifferentiated carcinoma. Among them, well-moderately differentiated was 25 and poorly differentiated was 11 cases respectively. There were 16 cases of T1 stage, 10 cases of T2 stage and 10 cases of T3 stage by the UICC standard. Lymph node metastasis was seen in 33 cases. Non- lymph node metastasis was seen in 3 cases. The patients contained 23 males

Table 1 Primers sequences of p53 gene exons 5-8

Primers	Sequences	
	Sense	Anti-sense
Exon 5	5'-TGT TCA CTT GTG CCC TGA CT-3'	5'-CAG CCC TGT CGT CTC TCC AG-3'
Exon 6	5'-ACA GGG CTG GTT GCC CAG GGT-3'	5'-CTC CCAGAG ACC CCA GTT GC-3'
Exon 7	5'-GGT CTC CCC AAG GCG CAC TGG-3'	5'-AGG CTG GGG GCA CAG CAG GCC-3'
Exon 8	5'-ATT TCC TTA CTG CCT CTT GC-3'	5'-AAG TGA ATC TGA GGC ATA AC-3'

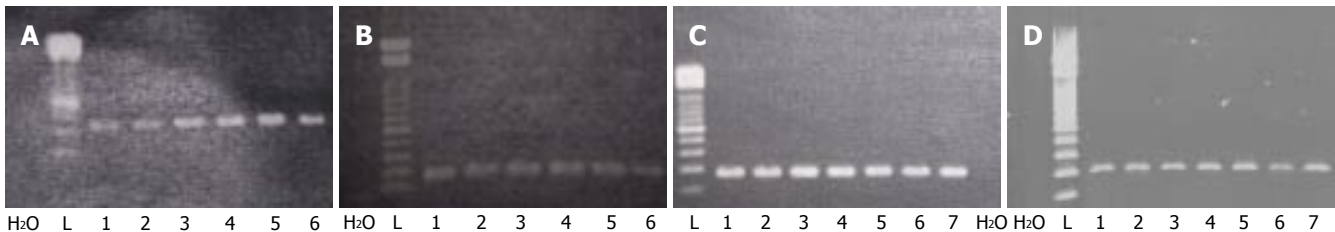


Figure 1 p53 exons 5, 6, 7, 8 PCR product. L : 100 bp DNA ladder; 1-7: Samples. A: Exons 5; B: Exons 6; C: Exons 7; D: Exons 8.

and 13 females, with age ranging from 36 to 71 (median, 61.2) years. All of the samples were frozen at  $-80^{\circ}\text{C}$  until DNA extraction and subjected to histological diagnosis by a pathologist.

#### DNA extraction

DNA was extracted from tissues using a QIAamp DNA Micro kit: QIA (Germany). Tissue samples weighing less than 10 mg were placed into a 1.5 mL microcentrifuge tube. Immediately 180  $\mu\text{L}$  buffer ATL and 20  $\mu\text{L}$  proteinase K were added and mixed by pulse-vortexing for 15 s. Then they were incubated at  $56^{\circ}\text{C}$  overnight. Two hundred microliter buffer AL and 200  $\mu\text{L}$  ethanol (100%) were added and incubated for 5 min at room temperature. After that, all of the lysates were applied onto the QIAamp MinElute column. Five hundred microliter buffer AW1 and buffer AW2 were added. After centrifugation, 100  $\mu\text{L}$  buffer AE was applied to get DNA. DNA quantity was determined by the ratio of A260/280.

#### Primer sequences and PCR amplification

Table 1 shows primer sequences used for p53 exons 5-8, which was synthesized by Hokkaido Bioscience Co. (Japan). PCR used a 20  $\mu\text{L}$  reaction volume containing 1 unit of Hot start EXTAq DNA polymerase (Takara, Biochemical, Japan), 2  $\mu\text{L}$  of  $10 \times$  EXTAq buffer, 2  $\mu\text{L}$  of dNTP mixture and each primer (8 pmol each for reaction) and 1  $\mu\text{L}$  of DNA template. The condition of the first PCR is as follows:  $96^{\circ}\text{C}$  for 3 min for denaturation, 40 cycles of  $96^{\circ}\text{C}$  for 30 s,  $60^{\circ}\text{C}$  for 30 s,  $72^{\circ}\text{C}$  for 30 s with a final elongation step of 4 min at  $72^{\circ}\text{C}$ . Water was used as a negative control. Five microliters of PCR product were analyzed on 1% TBE gel electrophoresis. Each sample was repeated three times.

#### DNA sequencing

All of the PCR products were purified using Auto seq TMG-50 (Amersham Biochemical Company, USA). Big-Dye Terminator Cycle sequencing Ready Reaction (Perkin Elmer, USA) was used. The primers of sequencing were

the same as PCR primers. But its concentration was one tenth of PCR primers. The condition is as follows:  $95^{\circ}\text{C}$  for 4 min,  $95^{\circ}\text{C}$  for 30 s,  $55^{\circ}\text{C}$  for 30 s,  $72^{\circ}\text{C}$  for 30 s for 40 cycles with a final step of 4 min at  $72^{\circ}\text{C}$ . Both sense and antisense chains were analyzed on an ABI prism 310 Genetic Analyzer (Perkin Elmer). Each sample was repeated three times.

#### Immunohistochemistry

Five-micron sections were dewaxed in xylene and rehydrated. Endogenous peroxidase was destroyed by a 15-min treatment in 30 mL/L hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) in phosphate-buffered saline (PBS) at room temperature. The sections were blocked with a combination of normal mouse serum and then incubated with anti-p53 protein (dilution 1:50, mouse anti-p53 protein by Boster Co.), followed by biotinylated-conjugated sheep anti-mouse IgG (Boster Co.). The complex was visualized by diaminobenzidine (Boster Co.). The specificity of the reaction was confirmed by use of negative control, blank control and substitution control, in which PBS substituted the secondary antibody biotinylated-conjugated sheep anti-mouse IgG.

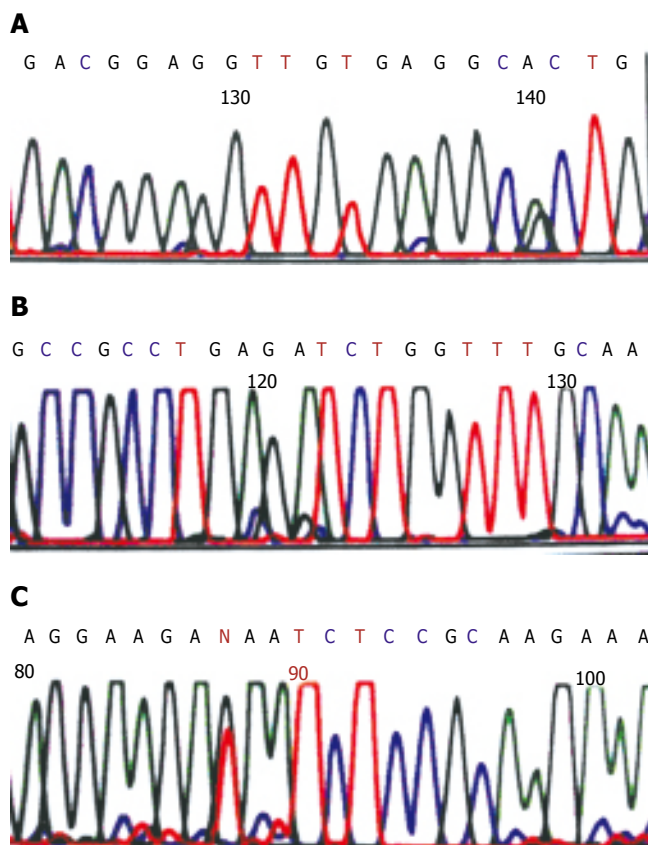
#### Statistical analysis

The results were analyzed with  $\chi^2$  test.  $P < 0.05$  was taken as significant.

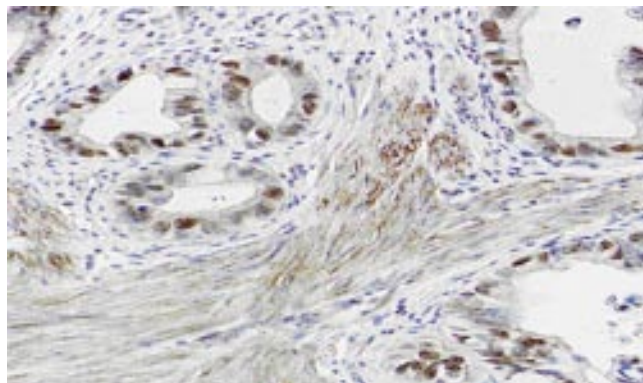
## RESULTS

#### p53 gene mutations and expression of P53 protein in cholangiocarcinoma tissues

Positive band of exons 5-8 was found in all samples after PCR amplification (Figures 1 A-D). p53 gene mutations were detected in 22 of 36 patients (61.1%) by DNA sequencing. Among them, there were 7 cases of exon 5 mutations, which were located on 161, 175 and 196 codons. All were transition (G:C/A:T). Six cases of exon 6 mutations were located on 209, 213 and 215 codons, of which 4 cases were of transition (G:C/A:T) and 2 cases



**Figure 2** Tranversion of *p53* exons 5, 6, 8 in cholangiocarcinoma. **A:** Exons 5 (G:C/A:T); **B:** Exons 6 (G:C/A:T); **C:** Exons 8 (G→T).



**Figure 3** P53 protein was positive in cholangiocarcinoma (SABC × 400).

of tranversion (G-T). Three cases of exon 7 mutations were located on 248, 252 codons. Six cases of exon 8 mutations were located on 282, 287, 289, 306 codons, of which 4 cases were of transition (G:C/A:T) and 2 cases of tranversion (G-T) (Figures 2 A-C).

Nineteen cases (52.8%) were positive for P53 protein. P53 protein localized in the nuclei of cholangic epithelial cells. Moreover, it was crisp and finely granular (Figure 3).

#### Correlation of *p53* gene mutation and clinical parameters

There were significant differences in degree of differentiation and invasion between the positive and negative samples of P53 protein expression ( $P < 0.05$ ,  $P$

**Table 2** Correlation of mutation status of *p53* gene and P53 protein expression with pathologic parameters in cholangiocarcinoma

Pathologic character	n	<i>p53</i> gene mutation		P53 protein expression	
		Positive (n, %)	P value	Positive (n, %)	P value
<b>Pathologic type</b>					
Tubular carcinoma	18	11 (61.1)		10 (55.6)	
Papillary carcinoma	9	6 (66.7)		5 (55.6)	
Mucoid carcinoma	4	2 (50.0)		2 (50.0)	
Non-differentiated carcinoma	5	3 (60.0)	> 0.05	2 (40.0)	> 0.05
<b>Differentiation</b>					
Well-Moderate	25	13 (52.0)		9 (36.0)	
Poor	11	9 (81.8)	> 0.05	10 (90.9)	< 0.05 <sup>a</sup>
<b>Invasion</b>					
T1	16	7 (43.8)		2 (12.5)	
T2	10	8 (80.0)		8 (80.0)	
T3	10	7 (70.0)	> 0.05	9 (90.0)	< 0.05 <sup>c</sup>
<b>Lymph node metastasis</b>					
N0	3	2 (66.7)		0 (0)	
N1	33	20 (60.6)	> 0.05	19 (57.5)	> 0.05

< 0.05). However, there were no significant differences in age, gender, degree of differentiation and invasion, lymph node metastasis, stage between the mutations and non-mutations ( $P > 0.05$ ) (Table 2).

## DISCUSSION

Cholangiocarcinoma is the second most common cancer of the hepatobiliary system. In recent years, the incidence and mortality of cholangiocarcinoma have been increasing in China<sup>[18,19]</sup>. In most patients, the disease is only diagnosed at a late stage. Patients with obstructive jaundice are frequently at the advanced stage of the disease, which is contraindicated for operation<sup>[20-26]</sup>. The development of molecular biology, the identification of molecular factors involved in cholangiocarcinoma carcinogenesis, and the elucidation of the mechanisms will significantly impact prevention, diagnosis, treatment and prognosis. *p53* gene is located on the short arm of chromosome 17, which consists of 11 exons. There are four mutation hot-spots (132-143, 174-179, 236-248, 272-281) within the core domain (exon 5-8), which are the key sites of biological activity of P53 protein<sup>[27,28]</sup>. *p53* gene is an important regulator factor of cell proliferation. It is related to cell cycles, DNA repair, cell differentiation and apoptosis. In the presence of DNA damage the expression of *p53* is enhanced and induces G1 cell cycle arrest until DNA is repaired. If repair is insufficient, *p53* gene promotes apoptotic cell death. However, *p53* gene mutation cannot block cell proliferation. Through cooperation with inactivation of tumor suppressor genes and activation of oncogenes, cells transform into malignant ones and become a tumor. *p53* tumor suppressor gene is the most common mutated gene in human cancer and is frequently seen in cholangiocarcinoma. Recent studies have found that positive expression of P53 protein is related to invasion and lymph node metastasis in cholangiocarcinoma<sup>[15,16]</sup>.

*p53* gene mutation could act as an index to estimate the prognosis of cholangiocarcinoma.

We examined mutation status of *p53* gene exons 5-8 by automated sequencing in 36 cases of cholangiocarcinoma. We found *p53* gene mutations in 22 of 36 (61.1 %) patients. Nineteen of 36 (52.8%) patients were positive for P53 protein expression. There were significant differences in extent of differentiation and invasion between positive and negative expression of P53 protein. However, there were no significant differences in age, gender, extent of differentiation, invasion, lymph node metastasis, and stage between the mutations and non-mutations.

Wild-type *p53* (non-mutated) has a short half-life of about 20 min. Mutant P53 protein has a greater stability with half-life prolonged up to 1.4-7 h. It can be detected by IHC method. But the use of different P53 antibodies and methods can result in a marked difference in the degree of overexpression, and varying levels of overexpression may also be noted in the same tumor specimen. IHC has been shown to have discordancy rates of 30%-35% compared with DNA sequencing. Thus, the determination of *p53* overexpression is not an accurate measure of *p53* function. Although DNA sequence analysis is a cumbersome, time-consuming and difficult method on archived material, it could provide a more accurate means of detecting *p53* mutations. Thus, we think *p53* gene mutation could not act as an independent index to estimate the prognosis of cholangiocarcinoma.

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