K-*ras* ONCOGENE AND *p53* GENE MUTATIONS IN CHOLANGIOCARCINOMA FROM THAI PATIENTS

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Abstract. Paraffin embedded tisues from twenty Thai patients with intrahepatic cholangiocarcinomas were studied for K-*ras* gene mutations at codon 12, 13 and 61 and for *p53* gene mutations in exon 5 to 8 using polymerase chain reaction and thermal cycle sequencing. Results showed that point mutations at these regions in K-*ras* oncogene were not present in all the samples. One case harbored a *p53* gene mutation in codon 282 in exon 8, CGG (arginine) to TGG (tryptophan), but the mutation was not found in other patient's tissues with similar histological features.

INTRODUCTION

Cholangiocarcinoma, a bile duct cancer, is one of the more common cancers in Southeast Asian countries (Sririsinha, 1994). In Thailand, the highest incidence of cholangiocarcinoma has been found in the northeast where there is also a high occurrence of liver fluke, *Opisthorchis viverrini*, infection. This correlation between high incidence rate of cholangiocarcinoma in the northeastern provinces of Thailand and endemicity of liver fluke infection has led to the suggestion of a causative association of this cancer in man with liver fluke infection (Sithithaworn *et al*, 1994).

Genes involved in cancer include both oncogenes and tumor suppressor genes (Weinberg, 1994). *Ras* genes are members of a family of oncogenes that is frequently found in cancer cells, with more than 80% of cancers harboring these mutant genes (Bos, 1989). The *ras* family consists of the three genes, H-*ras*, K-*ras* and N-*ras* which encode highly similar GTP-binding (G-) proteins (Barbicid, 1979). Mutation in the K-*ras* gene at codons 12, 13 or 61 is mostly frequently found in several type of cancers (Bos, 1989). *p53* is a tumor suppressor gene also frequently found mutated in a variety of human cancers (Hollstein *et al*, 1991; Greenblatt *et al*, 1994). In normal cells, wild type *p53* protein functions as a suppressor of cell proliferation and inhibits malignant transformation (Soussi *et al*, 1990). In the event of DNA damage, wild type *p53* is induced and leads either to cell cycle arrest or program cell death (Apoptosis) (Canman *et al*, 1994).

In this study, we have analysed K-*ras* oncogene and *p53* tumor suppressor gene using PCR-SSCP and direct sequencing from twenty Thai patients with cholangiocarcinoma.

MATERIALS AND METHODS

Tumor specimens

Twenty-three specimens from twenty Thai patients with cholangiocarcinoma were obtained from National Cancer Institute, Thailand. Serial sections of 3 μm thickness were made of the formalin-fixed and paraffin-embedded samples. One such section was stained with hematoxylin-eosin and examined microscopically for the histological features using criteria previously described (Nakajima *et al*, 1988). An adjacent section of 10 μm was used for DNA extraction.

DNA extraction

DNA was extracted from the tissue specimens by proteinase K digestion followed by phenol-choloform extraction as previously described. Tissue section was incubated in lysis buffer (10mM Tris-HCl pH8.3, 50mM KCl, 2.5 mM $MgCl_2$, 0.45% v/v Tween-20) containing 0.5 mg/ml proteinase K for 2 hours at 60°C (Tada *et al*, 1990). Protein was removed by phenol-chloroform (1:1 v/

v) solution according to standard protocol. The aqueous phase containing DNA was stored at 4°C prior to use.

Detection of K-*ras* gene mutation

Exon I of K-*ras* gene position 6381 to 6485 from codon 1 to 39 was amplified using the polymerase chain reaction (PCR) with the primers *KR5* (5' GACTGAATATAAACTTGTGG 3', sense strand position 6381 to 6400) and *KR3*(5' CTATTGTTGGATCATATTCG 3', antisense strand, position 6485 to 6465). The reaction mixture contained 50-100 ng DNA, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.002% gelatin, 2.5mM $MgCl_2$, 200 μM of each dNTP, 1 μM of each primer and 2.5 U *Taq* DNA polymerase (Perkin-Elmer Co, USA). The reaction was performed in DNA Thermal cycle 480 (Perkin Elmer Co, USA) for 50 cycles (each cycle consisting of 95°C for 1 minute 52°C for 1 minute and 72°C for 1 minute. K-*ras* gene exon II, position 19459 to 19603, was also amplified using the primers *K5* (5' CCCTTCTGACGATTCCTACA 3', sense strand, position 19459 to 19478) *K3* (5' TACACAAAGAAAGCCCTCCC 3', antisense strand position 19603 to 19584), using the same thermal cycling condition as described above. The nucleotide mutations at codons 12 and 13 in exon 1 and at codon 61 in exon 2 of K-*ras* gene were determined from the PCR products by thermal cycle sequencing (AmpliCycle™ sequencing kit, Perkin-Elmer Co, USA) employing KR3' and KR5' as sequencing primers for exon 1 and 2, respectively.

Identification of *p53* gene mutation

The gene mutations in *p53*, exons 5 to 8, were identified by PCR-SSCP and thermal sequencing. For PCR-SSCP, 10 μl reaction volume contained 100 ng DNA, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1% Triton X-100, 25 μM of each dNTP, 1μCi of α-^{32}p dCTP (3,000 Ci/mmol, Amersham, Inc UK), 1 μM of each primers and 1 U *Taq* DNA polymerase. The sequences of these primers together with $MgCl_2$ concentration are shown in Table 1. PCR was performed in DNA Thermal Cycler 480 for 30 cycles (each cycle consisting of 95°C for 1 minute, 57°C for 1 minute for exons 5 and 7 and 55°C for 1 minute for exons 6 and 8; and 72°C for 1 minute). The PCR products were electrophoresed in 6% polyacrylamide containing 5% glycerol at 400 V for 7 hours. The migration of DNA strands in the gel were detected by autoradiography. The nucleotide sequences of *p53* gene in exons 5 to 8 from all patients were determined by AmpliCycle™ sequencing kit with sense or antisense primer as the sequencing primer. The reactions were performed by the method described by the manufacturer.

RESULTS

Histology of cholangiocarcinoma

Twenty patients with cholangiocarcinoma consisted of 14 males and 6 females with age ranging from 34 to 64 years (mean 51 years). Histological features and percentage of cancerous cells in the tissues are shown in Table 2. A number of patients

Table 1

PCR primers and $MgCl_2$ concentration used for the amplification of *p53* genes in exons 5 to 8.

Exon	Primer	Nucleotide position	$MgCl_2$, mM
5	F 5' TTCCTACAGTACTCCCCTGC 3' R 5' AGCTGCTCACCATCGCTATC 3'	13046-13248	57
6	F 5' CCTCTGATTCCTCACTGATT 3' R 5' TTGCAAACCAGACCTCAGGC 3'	13292-13445	55
7	F 5' GTGTTATCTCCTAGGTTGGC 3' R 5' TCCTGACCTGGAGAGTCTTCCA 3'	13986-14116	57
8	F 5' CCTGAGTAGTGGTAATCTAC 3' R 5' GCTTGCTTACCTCGCTTAGT 3'	14443-14598	55

F = sense strand primer
R = antisense strand primer

exhibited more than one type of histology : in patient no. 10, a well differentiated tubular adenocarcinoma (WDC) was found in one tissue sample whereas another tissue sample showed moderately-well differentiated tubular adenocarcinoma (MWDC), patient no. 13 showed well differentiated papillary adenocarcinoma (WDC(P)) type in one tissue sample and MWDC type in another sample; and one tissue sample of patient no. 17 contained well differentiated adenocarcinoma whereas moderately-well differentiated adenocarcinoma was found in another tissue sample. The percentage of cancerous cells in all tissues varied from 20 to 100%.

K-*ras* and *p53* gene mutations

The nucleotide sequence of K-*ras* around codons 12 and 13 in exon 1 and codon 61 in exon 2 were determined from DNA amplified from all patients. No gene mutations among these patients could be detected (data not shown). Mutations in exon 5 to 8 of *p53* gene were screened by PCR-SSCP. Fig 1 shows the abnormal mobility shift present in exon 8 of the *p53* gene from patient numbers 7 and 13, respectively. The sequence of this exon showed a nucleotide change in codon 282, from CGG (arginine) to TGG (tryptophan) in patient no. 13 (Fig 2) and normal sequence in patient No. 7 (data not shown). Histological feature of this patient was well-differentiated papillary adenocarcinoma (Table 2). However, no mutation in this region of the gene could be found in other patients with similar histological features (patients no.3 and 19).

Table 2

Histological type from 20 Thai patients cholangiocarcinoma.

Patient no.	Age (yr)/ sex	Histological type	percent cancerous cell in a tissue specimen
1	52/F	WDC	30
2	56/F	WDC	90
3	61/M	WDC(P)	60
4	55/M	MDC	90
5	39/M	MDC	90
6	52/F	MWDC	95
7	45/M	WDC	20
8	38/M	MDC	60
9	55/M	MDC	95
10	55/M	WDC; MWDC	95
11	53/M	MDC	95
12	64/M	MDC	90
13	34/M	WDC(P); MWDC	90
14	47/M	MDC	100
15	55/F	MDC	80
16	59/M	MDC	50
17	46/F	WDC; MWDC	90
18	52/F	MDC	100
19	47/M	WDC(P); MWDC	80
20	56/M	MWDC	90

MDC = Moderately differentiated tubular adenocarcinoma
WDC = Well differentiated tubular adenocarcinoma
MWDC = Moderate-well differetiated tubular adenocarcinoma
WDC(P) = Well differentiated papillary adenocarcinoma

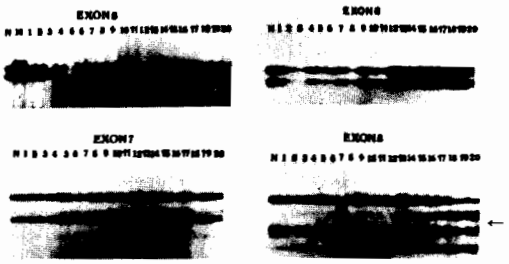

Fig 1–PCR-SSCP analysis for *p53* gene mutation in exons 5 to 8 of DNA extracted from cancerous tissue of 20 patients with cholangiocarcinoma. PCR-SSCP analysis was performed as described in Materials and Methods. The PCR products were diluted 1:10 with stop solution and heated at 95°C for 5 minutes. One microlitre aliquoit was loaded onto 6% polyacrylamide-5% glycerol gel in 0.5xTBE buffer and electrophoresed at 400 V at 25°C for 7 hours, followed by autoradiography overnight. The arrow indicates an abnormal mobility shift in exon 8.
Lane N = leukocyte DNA from normal individual
Lane M = DNA from sample with known mutation in exon 5.
Lanes 1-20 = DNA from cancerous tissues of 20 patients.

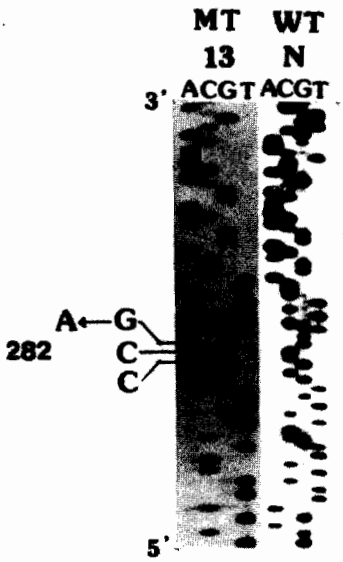

Fig 2--Autoradiogram of nucleotide sequencing gel of *p53* gene at codon 282 from cancerous tissue of patient no.13. The nucleotide sequence of *p53* gene in exon 8 from patients no. 13 and normal individual (N) were determined using an antisense primer as a sequencing primer. A base change from CC*G* (normal DNA) to CC*A* (patient) at codon 282 in the antisense DNA strand.

DISCUSSION

Cholangiocarcinoma arising from the epithelium of intrahepatic bile duct often develops following exposure with either colloidal thorium dioxide (Thorotrast) (Sugihara and Kojiro, 1987), clonorchiasis (Schwatz, 1980) or opisthorchiasis (Shirai *et al*, 1992). However, a high frequency of K-*ras* mutation (9 out of 12 cases) and *p53* gene mutations (4 in 12 cases) in cholangiocarcinoma from Japanese patients whose past history shows no association with those factors has been reported (Tada *et al*, 1992; Kiba *et al*, 1993).

Animal experiments have shown that liver fluke induces inflammatory and proliferative changes in the liver of infected hamster, and cholangiocarcinoma can only be induced in hamsters infected with liver fluke together with nitrite or dimethylnitrosamine (Thamavit *et al*, 1978, 1987, 1993). These results correlate with epidemiological studies showing a high frequency of cholangiocarcinoma in Northeast Thailand, an endemic area of liver fluke, *Opisthorchis viverrini*, infection (Sithithaworn *et al*, 1994) and where the native foodstuff in these area contain high levels of nitrate and nitrite (Migasena, 1994).

In this study, mutations of K-*ras* gene in Thai cholangiocarcinomas could not be detected in different histological types of samples. This observation is the same as previous reports in Thai patients with cholangiocarcinoma (Tsuda *et al*, 1992; Kiba *et al*, 1993).

It has been shown that more than 30% of cases in Thai and Japanese cholangiocarcinoma harbored the *p53* gene mutation (Kiba *et al*, 1993). In this study, this mutation was found only in patient no. 13 whose tissue histology type was well differentiated papillary adenocarcinoma however, two other patients with the same histology type (no. 3 and 19) did not carry the *p53* gene mutation.

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