The Incidence of K-*ras* Codon 12 Mutations in Cholangiocarcinoma Detected by Polymerase Chain Reaction Technique

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Abstract

In the study, to analyse a K-*ras* oncogene mutated at codon 12 in 24 patients with cholangiocarcinomas, four (16.67%) of them contained this point mutation. One of 4 was peripheral and the others were hilar tumors. There was no significant relationship between mutation and clinical features in terms of age, sex, endemic area, tumor location, tumor grading and pathological features. In our study, the incidence of K-*ras* codon 12 mutation in Thai patients with cholangiocarcinoma was lower than that found in British and Japanese patients. The discrepancy of incidence and type of the mutations, in different races and environment probably indicates that there is/are different etiologic mechanism(s) in the pathogenesis of cholangiocarcinoma.

The *ras* gene is one of the most common oncogenes found in human cancer[1]. This gene encodes a related protein, Mr 21,000 which is composed of 188-189 amino acid residues. The Mr 21,000 has an important role in controlling the mechanism of cellular growth and differentiation[1,2]. Activated oncogenes in the *ras*-family can be H- *ras*, K- *ras*, or N-*ras*; and the point mutation in codon 12, 13, 61 is the most mutation mechanism[1]. The origin and pathological mechanism of periampullary carcinoma remains a challenging issue to surgical oncologist and pathologists who are getting involved. There has been a report of a high prevelence of K-*ras* mutation in pancreatic adenocarcinoma and cholangiocarcinoma[3-6], but less frequency in ampullary or duodenal carcinoma. Presence or absence of this mutation might be useful in the explanation of why and how periampullary carcinoma develops. With the incidence of 318 : 100,000 cholangiocarci-

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noma is the most common cancer in the northeastern Thailand[7]. Some experimental studies have also supported the close relationship between *Opisthorchiasis viverrini* infection and cholangiocarcinoma[8,9]. We detected the point mutation by using polymerase chain reaction procedure based on mismatched primers[10,11]. We also tested 24 cholangiocarcinomas, the presence of the *ras* gene mutation and clinical and pathologic features of this type of tumor.

MATERIAL AND METHOD

Patients and Tissue sources

Twenty-four resected specimens of cholangiocarcinoma were obtained from the Department of Surgery, Vajira Hospital. The specimens were fixed in 10 per cent buffered formalin, embedded in paraffin wax at 56°C, then stored at room temperature. A serial section of 8 μm thickness was performed, then stained with hematoxylin-eosin, and examined microscopically. The histological diagnosis of chloangiocarcinoma was confirmed in each patient.

Polymerase chain reaction (PCR)

DNA preparation : DNA was prepared from a single 8 μm tissue section with histological confirmation of the presence of cholangiocarcinoma of adjacent sections. The procedure to extract and amplify DNA from paraffin-embedded tissue was as described previously[11,12]. The section was incubated in 200 μl of lysis buffer (10 mM Tris HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.45% Tween-20) containing proteinase K (0.5 mg/ml) for 2 h at 55°C, with regular shaking. The mixture was then boiled at 100°C for 10 min, and 20 μl of this preparation was used as the substrate for DNA amplification. The remaining mixture was stored at 4°C after addition of 1.0 mM EDTA; this preparation remained a useful source of genomic template for 4 to 6 weeks.

This method for DNA preparation avoids the use of xylene and ethanol to remove paraffin from the tissue section. Paraffin melts and floats to the top during the 55°C incubation and boiling steps. The method enables a large number of amplifications to be performed from DNA from a single section, which is especially useful when the tissue is in limited supply.

PCR: The templated DNA was specifically amplified at the K-*ras* codon 12. The PCR product was 157-base pairs in length. (Fig. 1) The primers used to effect the PCR product haboring the K-*ras* gene were 5'-ACTGAATATAAACTTGTGG TAGTTGGACCT-3' as a forward primer and 5/-TCAAAGAATGGTCCTGGACC-3' as a reverse primer (underlined bases represent mismatches from the K-*ras* DNA sequence). Amplifications with *Taq* polymerase were performed in 50 μl reaction mixtures containing 2 units of *Taq* polymerase, 50 pmol of each primer, deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, dTTP) at 50 μM, 2.0 mM Mg^{2+}, 60 mM KCl, and 10 mM Tris-HCl (*p*H 8.8). The reaction mixtures were overlaid with 75 μl of mineral oil and then subjected to amplification. Each cycle comprised 94°C for 1 min, 50°C for 1 min, and 72°C for 0.5 min. Total number of cycles used in the PCRs was 40, followed by a *Bst*N I digestion. A negative (no DNA) control was run parallel with each PCR analysis.

Restriction Fragment Length Polymorphism Analysis

Restriction enzyme digests with *Bst*N I were performed using the conditions recommended by the manufacturer (New England Biolabs, Beverly, MA). After amplification, the DNA was resolved by electrophoresis in agarose gel[13].

Statistical Method

The Fisher's exact test, Student's *t*-test and χ^2 *for trend* with 95 per cent of confidence interval were used in this study.

RESULTS

Demographic / Pathological data

The patients enrolled in this study were 13 men and 11 women (age range: 33-89). The location of the tumors were 4 cases of hilar type and 20 cases of peripheral type. Papillary histology was found in 3, and tubular pattern in 21. According to grading of the tumor, 12 were poorly, 9 were moderately and 3 were well-differentiated histology. Only 4 (16.67%) out of 24 cholangiocarcinomas were positive for the point mutation of K-*ras* codon 12; which were demonstrated as three bands (lane 2, 3, 4) shown in Fig. 2.

Primers containing mismatches can be effectively used for PCR amplification. It has been also provided that the mismatch is not at the extreme 3' end of the primer[14]. Jiang[15] and Levi[11] used this technology to detect K-*ras*

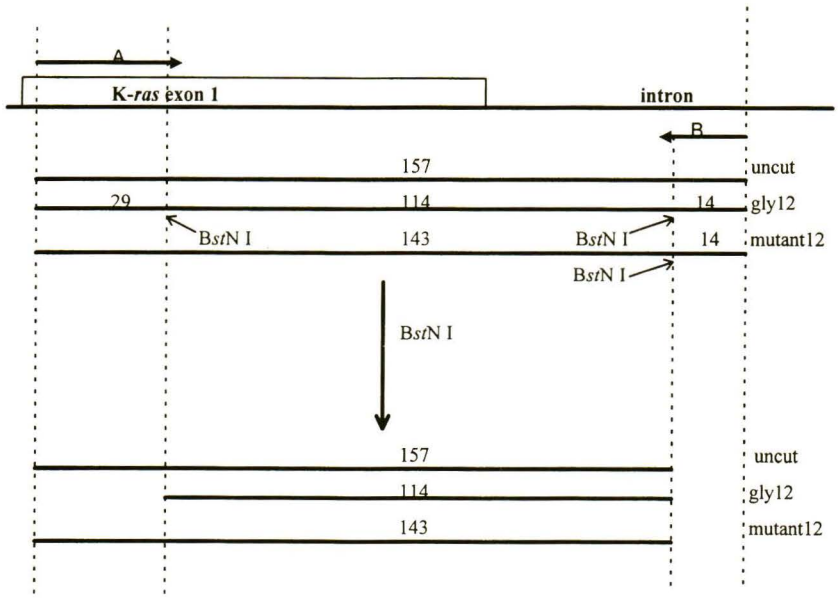

Fig. 1. Schematic diagram showing the consequences of PCR with the primers indicated. PCR with Primers A and B gives rise to a 157-base pair fragment containing two *Bst*N I restriction sites if codon 12 is normal (gly 12) and just one *Bst*N I site if codon 12 contains a mutation in either of its first two bases. Therefore, wild-type fragments cleave to yield 29, 114, and 14-base pair products. The symbol on the primer indicates a mismatch from the genomic normal K-*ras* sequence, which gives rise to a *Bst*N I restriction site under the conditions indicated.

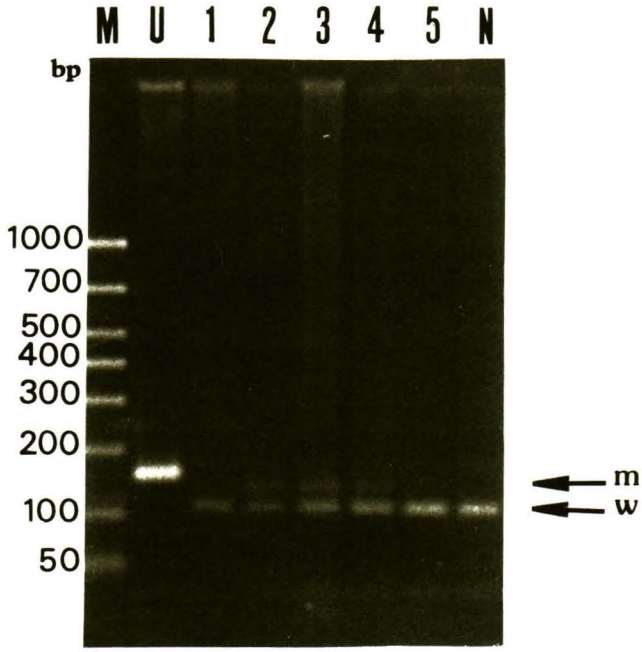

Fig. 2. Detection of mutation in the K-*ras* codon 12 in cholangiocarcinoma by PCR based on mis-matched primers. Mobility shifts were detected in lane 2, 3, 4 (143-base pair of PCR product). Lane M, fragment mixture from cleavage of plasmid pBR322 DNA with HaeIII. Lane U indicates uncut. Arrows (right) indicate the positions on the mutant (m) and wild type (w) bands.

codon 12 mutations. Using a primer incorporation a C residue at the first position of codon 11, PCR amplification of the normal allele created a *Bst*N I restriction enzyme cleavage site (CCTGG) overlapping the first two nucleotides of codon 12. Amplification of K-*ras* mutant at either of the first two positions of codon 12 does not create this *Bst*N I site (Fig. 1). The PCR product resulted from Primers A (forward primer) and B (reverse primer) which were subsequently digested with *Bst*N I consisting of 114- and 143-base pair bands. The 143-base pair band, diagnostic of mutation in K-*ras* codon 12, was still detectable in the 1:10 dilution, a sensitivity similar to the 1/16 figure obtained by Jiang[15]. This is because of annealing a mixture of normal and mutant DNA, heterohybrids form which the enzyme is unable to cleave.

The amplified product consisted of the original genomic template, mutant fragments which resisted digestion by *Bst*N I, uncut wild-type fragments (left undigested by *Bst*N I) and linearly amplified PCR fragments which failed to incorporate the mismatch in Primer A. The ability of this modified technique is to detect one cell heterozyous for K-*ras* codon 12 mutation in the presence of over 500 normal cells and is equivalent to a sensitivity of one mutant allele in the presence of 1000 normal alleles[16].

DISCUSSION

Not only the incidence, but also the spectrum, of *ras* gene mutations vary in a wide range in human cancers[2,18]. These genes are converted to active oncogenes by point mutations occurring in either codon 12, 13, or 61[1]. By far, the highest observed frequency of K-*ras* codon 12 mutation occurred in carcinoma of the pancreas[3-6], colon[17-19] and lung[20]. On the contrary, the frequency of the mutations in gastric cancer[18] and hepatoma[12] are extremely low. The occurrence of K-*ras* mutation in bile duct carcinoma was widely different in various studies.

When we compared 4 cases of K-*ras* codon 12 mutations and 20 cases without the

Table 1. Clinico-pathological features of 24 patients with cholangiocarcinoma and presentation of K-*ras* codon 12 mutation.

Patient No.	Age / sex	Cholangiocarcinoma							Mutation codon 12
		Type	Grade	Histology	Fibrous	Mucin	N*	L†	
1	64F	Hilar	moderate	Tubular	+	-	-	-	+
2	68M	Peripheral	poor	Tubular	-	-	-	-	+
3	68F	Hilar	poor	Tubular	-	-	-	-	+
4	58M	Hilar	poor	Tubular	+	+	-	-	+
5	43M	Peripheral	poor	Tubular	+	-	-	-	-
6	76F	Hilar	moderate	Tubular	-	-	-	-	-
7	57M	Peripheral	moderate	Tubular	-	-	-	-	-
8	55F	Peripheral	moderate	Tubular	+	+	+	+	-
9	73F	Hilar	moderate	Tubular	+	+	+	+	-
10	71M	Hilar	poor	Tubular	+	+	+	-	-
11	58F	Hilar	poor	Tubular	+	+	+	-	-
12	58M	Hilar	poor	Tubular	+	+	-	-	-
13	62F	Hilar	poor	Tubular	-	-	-	-	-
14	72M	Hilar	poor	Tubular	+	+	-	-	-
15	33F	Hilar	well	Papillary	+	+	+	+	-
16	46F	Hilar	well	Papillary	-	+	-	-	-
17	89M	Hilar	poor	Tubular	+	+	-	-	-
18	64F	Hilar	poor	Tubular	+	-	+	-	-
19	48M	Hilar	moderate	Tubular	+	+	+	-	-
20	46M	Hilar	moderate	Tubular	+	+	+	-	-
21	66M	Hilar	moderate	Tubular	+	+	+	+	-
22	41F	Hilar	well	Papillary	+	+	-	-	-
23	50M	Hilar	moderate	Tubular	-	+	+	-	-
24	50M	Hilar	poor	Tubular	+	+	+	-	-

Note : N* represented perineural invasion. L† represented lymphatic invasion.

Table 2. Comparing of clinical information between patients with and without K-*ras* codon 12 mutation.

Characteristics	K-*ras* codon 12		Probability* of exactly observed per mutation
	Mutation (4 cases)	No mutation (20 cases)	
M : F	2 : 2	11 : 9	0.385
Average age (yrs)	64.5±4.7	57.9±13.9	(0.364†)
Endemic area	1 (25%)	2 (10%)	0.375
Location of tumor			0.429
peripheral	1 (25%)	3 (15%)	
hilar	3 (75%)	17 (85%)	
Tumor grading			(0.193δ)
well	0	3 (15%)	
moderate	1 (25%)	9 (45%)	
poor	3 (75%)	8 (40%)	
Pathological features			
Fibrous streak	2 (50%)	16 (80%)	0.216
Mucin-producing	1 (25%)	15 (75%)	0.084
Perineural invasion	-	11 (55%)	0.067
Lymphatic invasion	-	4 (20%)	0.456

Note : * Fisher's exact test, † Two-tailed p-value Student's *t*-test., δ Two-tailed p-value (χ^2 *for trend* = 1.691, *df* = 1).

mutation (Table 2), there was no demonstrable correlation between K-*ras* codon 12 mutation and age, sex, location of tumor, tumor grading and some pathological features. Regarding the patients' age and sex, there was no significant difference in age distribution between the mutation and non-mutation groups, (64.5 ± 4.7 *vs* 57.9 ± 13.9 yrs, p= 0.364). The sex ratios between mutation and negative-mutation groups were similar 2:2 *vs* 11:9, p= 0.385. Our finding of no sex preference in mutation and non-mutation groups was the same as a previous study[21].

For K-*ras* codon 12 mutation correlated with histologic grading, our results showed that the prevelence of this mutation seemed to increase in poorly differentiated tumors. Nevertheless, the mutation *per se* did have no significant trend to be poorly differentiated tumor (χ^2 *for trend* = 1.6912, p = 0.193). Contrary to other studies, K-*ras* expression has been reported to be increased in less differentiated tumors[12,16,22]. In our series as well as previous reports[11,21], there was no demonstrable correlation between the mutation and pathological features of the tumor. Neither perineural nor lymphatic invasions characterized in poorly differentiated tumor were found in these 4 cases. Moreover, it is curious that the universal finding of codon 12 mutation is at such variable frequencies[11,12,14-16]. Although Levi[11] and Watanabe[21] reported the highest incidence of the *ras* mutations in cholangiocarcinoma, direct sequencing of those cases did not show a uniform pattern of base mutation.

The incidence of K-*ras* codon 12 mutations in cholangiocarcinoma vary in different studies. Location of tumor, racial difference, pre-existence of liver fluke infestation, exposure to carcinogenic agents and other environmental factors have been claimed to be part of the tumor development. The higher incidence has been reported in hilar cholangiocarcinoma[11,12,16,21,23]. In contrast, our series showed a higher incidence in peripheral type (25% compared to 15% in hilar type) as shown in Table 3. The incidence of K-*ras* codon 12 mutation was considerably lower in Thai patients (4% in Kiba's study[23], 17% in this study) than Japanese patients (33% - 100%)[12,16,21,23] or a British series (100%)[11]. Regarding the hilar cholangiocarcinoma, Thai patients also had a lower incidence (15%) of K-*ras* codon 12 mutation than Japanese and British patients (44%-100%)[11,12, 16,21]. Of note, Thai cholangiocarcinomas enrolled in Kiba's study[21] were from northeastern Thailand, which is well-known as an endemic area of the liver fluke, *O. viverrini*, and a high incidence of cholangiocarcinoma as well as cholangitis and

Table 3. The incidence of K-*ras* codon 12 mutation in different type of cholangiocarcinoma from previous studies compared with presented study.

Authors, year(Ref)	Patients' race	Peripheral		Hilar		Total	
		n	mutation	n	mutation	n	mutation
Tada, 1990[12]	Japanese	4	25%	5	80%	9	55.55%
Levi, 1991[11]	British	-	-	15	100%	15	100%
Tada, 1992[16]	Japanese	9	11.11%	9	44.44%	18	33.33%
Kiba, 1993[23]	Japanese	12	50%	-	-	12	50%
	Thai	26	3.85%	-	-	26	3.85%
Watanabe, 1994[21]	Japanese	-	-	10	100%	10	100%
This study, 1996	Thai	4	25%	20	15%	24	16.67%

cholangiohepatitis[24]. Whilst our patients were neither endemic residents nor opisthorchiasis carriers. This might implicate the different carcinogenic mechanism of cholangiocarcinoma even among a Thai group. An association between *O. viverrini* infestation and cholangiocarcinoma has been reported by studies using an animal model, but how it takes place is not yet known.

Furthermore, approximately 40 per cent of colon cancer showing K-*ras* gene mutations[17-19] might have known carcinogen as secondary bile acid in the stepwise progression of neoplastic cells to full malignancy. Secondary bile acids have been hypothesized to have a carcinogenic role in the pathogenesis of colon cancer[25]. By this pathway, degraded bile acids may act as a carcinogen for both colon cancer and cholangiocarcinoma.

Given that the incidence of K-*ras* gene mutation in biliary tract cancer is lower in Thai patients either from our study or Kiba's study than other groups, conjugated bile acids *per se* do not seem to evoke this mutation. However, there has not been a significant and definite shift of mutation pattern in K-*ras* gene codon 12 that might implicate racial or environmental factors. There was no association between the point mutation and histologic type or tumor location in Japanese *vs* Thai[23] and British *vs* Thai[11]. Our observations suggest that K-*ras* codon 12 mutation is not related to pathogenesis of cholangiocarcinomas in Thai patients, though data is limited. Kiba[23] reported Thai patients with intrahepatic cholangiocarcinoma carrying the high incidence of *p53* mutation which plays an important role in carcinogenesis of biliary tract in Thailand. Further investigation concerning the suppressor gene and oncogene is required, including its normal function and influence on the cell regulating system by its mutation.

The method of study might be also a major contributing factor detecting the mutation. We used a modified (single step) PCR based on mismatched primer which can detect one mutant allele in the presence of over 500 (0.2%) normal cells. Watanabe[21] claimed that using a modified two-step PCR method would boost the ability to detect a mutant allele up to 0.2 per cent, while direct subsequent to simple PCR can detect mutations if at least 20 per cent of the cell is present in the sample[21]. We believe that this assumption is possibly correct, however, maybe premature to conclude. A further comparison study of these two methods with the same sample is needed.

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อุบัติการณ์การกลายพันธุ์ของยีน-แรส ตำแหน่งที่ 12 ในมะเร็งท่อทางเดินน้ำดี ซึ่งตรวจพบโดยวิธีการเพิ่มปริมาณดีเอ็นเอ ด้วยปฏิกิริยาลูกโซ่

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ในการศึกษานี้ได้ทำการตรวจหาการกลายพันธุ์ของยีน-แรสตำแหน่งที่ 12 ในผู้ป่วยมะเร็งท่อทางเดินน้ำดี จำนวน 24 รายและพบว่า 4 รายที่มีการกลายพันธุ์ที่ตำแหน่งดังกล่าว. ผู้ป่วยหนึ่งในสี่รายนี้พบมะเร็งท่อทางเดินน้ำดีที่กลีบตับ, ส่วนที่เหลือสามรายเป็นมะเร็งที่บริเวณขั้วตับ จากการศึกษาความสัมพันธ์ระหว่าง อุบัติการณ์การกลายพันธุ์ของยีนแรสตำแหน่ง 12 กับข้อมูลทางคลินิก พบว่าไม่มีความสัมพันธ์กับ ปัจจัยดังต่อไปนี้ ได้แก่ อายุ, เพศ, ความรุนแรงของเซลมะเร็ง และ ลักษณะพยาธิสภาพอื่น ๆ. นอกจากนี้ได้ทำการเปรียบเทียบอุบัติการณ์ที่ได้จากการศึกษานี้กับรายงานอื่น ๆ พบว่าผู้ป่วยคนไทยมีอุบัติการณ์ การกลายพันธุ์ที่ตำแหน่งนี้น้อยกว่าผู้ป่วยชาวอังกฤษและชาวญี่ปุ่น ความแตกต่างของอุบัติการณ์นี้ อาจสืบเนื่องจากเชื้อชาติ, สิ่งแวดล้อม, ปัจจัยเสี่ยงของการเกิดมะเร็งและกลไกการเกิดมะเร็งท่อทางเดินน้ำดีในแต่ละเชื้อชาติ / ภูมิลำเนาที่แตกต่างกัน ส่งผลให้ชนิดของการกลายพันธ์ยีนแรสที่แตกต่างกันไปด้วย

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