CANCER GENES AND CHOLANGIOCARCINOMA

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Abstract. Genes involved in cancer development include oncogenes and tumor suppressor genes. *Ras* oncogene and mutations in *p53* tumor suppressor gene are commonly found in many types of cancer. In Thai patients with cholangiocarcinoma *ras* oncogenes occur less frequently than in other ethnic groups and furthermore, *p53* mutations also occur with lower incidence when compared with Japanese subjects. It is unclear at this time the basis for these differences.

INTRODUCTION

Cholangiocarcinoma, bile duct cancer, is one of the more common cancers in Southeast Asian countries (Sithithaworn *et al,* 1994; Sirisinha, 1994). The highest incidence has been found in Northeast Thailand which is also a high endemic area of liver fluke *(Opisthorchis viverrini)* infection. The age-standardized incidence rates of cholangiocarcinoma are 84.6 and 36.8 per 100,000 in males and females, respectively. On the other hand, a nonendemic area such as Chiang Mai, located in North Thailand, has an incidence rate of 6.1 and 4.8 per 100,000 in males and females, respectively. In Hong Kong, where another liver fluke, *Clonorchis sinensis* is present, the age-standardized incidence rates are 5.4 and 3.1 per 100,000 in males and females, respectively. In contrast, the lowest incidence rate is found in Western countries, a ~ 2 per 100,000.

The correlation between high incidence rate of cholangiocarcinoma in Northeastern provinces of Thailand and the endemicity of liver fluke infection has led to the suggestion of an association of this cancer with liver fluke infection in man. Animal experiments in which *O. viverrini* infected hamsters were fed with water containing dimethylnitrosamine resulted in the development of cholangiocarcinoma, whereas control groups which were not infected but treated only with dimethylnitrosamine or were infected with parasites but not treated did not develop cancer (Thamavit *et al,* 1978; 1993). Similar results were obtained with *C. sinensis*-infected hamsters (Lee *et al,* 1994). Therefore, these data suggest that dimethylnitrosamine plays a critical role in development of cholangiocarcinoma in hamsters associated with liver fluke infection. High levels of dimethylnitrosamine have been found in fermented food which form part of the diet among Northeastern Thai people (Migasena and Changbumrung, 1974).

ONCOGENES

Genes involved in cancer include oncogenes and tumor suppressor genes. In normal cells, proto-oncogenes encode proteins controlling cell growth and differertiation. Oncogenes can be classified into at least 4 groups depending on the function of their encoded proteins : cytoplasmic and membrane kinase, guanine nucleotide binding protein (G-protein), growth factor and receptor, and nuclear protein. Mutations in one or more of the oncogenes cause the change from normal to malignant state (Weinberg, 1994).

The *ras* genes are members of a family of oncogenes that is frequently found expressed in cancer cells, with more than 80% of cancers harboring *ras* mutants (Bos, 1989). The *ras* family consists of three genes, H-*ras,* K-*ras,* and N-*ras,* which encode highly similar GTP-binding (G-) proteins, consisting of 188 or 189 amino acids with a molecular weight of 21,000 (Barbicid, 1979). These proteins have also been known as *p21* proteins. In normal cells these G-proteins control adenylate cyclase activity involved in the transduction of external stimuli induced by growth factors or by factors involved in cell differentiation (Haubruck and McCormic, 1991). The *ras* genes are converted to active oncogenes by single point mutations at codons 12, 13 or 61 (Barbicid, 1979). Changes in the protein structure by amino acid substitution at these positions cause an inhibition of the intrinsic GTPase activity of ras proteins leading to the loss of their function in controlling cell differentiation.

Mutations of *ras* genes have been identified by employing techniques involving polymerase chain reaction. Nucleotide substitution in codon 12, 13 or 61 can be determined from amplified DNA products using methods such as single strand conformation

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polymorphism (SSCP) (Suzuki *et al,* 1991), mutant allele-specific oligonucleotide (MASO) hybridization (der Vries *et al,* 1986), direct sequencing (McMahon *et al,* 1987) or primer introduced restriction analysis (PIRA) (Levi *et al,* 1991). The latter method is able to detect mutations in less than 10% of cancerous cells in tissue specimens, whereas the direct sequencing method detects mutation in tissue containing > 20% of the malignant cells (Cheng and Haas, 1992).

The *ras* gene mutation in cholangiocarcinoma has been studied in tissue specimens obtained from hospital patients. The frequency of *ras* gene mutations detected from different ethnic groups is summarized in Table 1 and the spectrum of mutation in Table 2, with reference sources.

In extrahepatic bile duct carcinoma from 15 English patients with K-*ras* codon 12 mutations, 6 cases contained a single mutation: (4 with GGT (glycine) to GGT (valine), one had GGT to GAT (aspartic) and one had GGT to AGT (serine)), eight cases had bimutations and one case had three mutations altogether.

A K-*ras* gene codon 12 mutation (GGT to GAT) was detected in 2 of 3 patients with cholangiocarcinoma in the USA. The mutation identified from this tumor was different from that found in the stool and duct lesions showing hyperplasia from the same patients. In Japanese patients, 1 out of 2 extrahepatic bile duct cancers harbored a K-*ras* gene mutation at codon 12 (GGT to AGT). Among intrahepatic bile duct cancer from 30 Japanese patients, K-*ras* codon 12 mutations were found in 13 cases, with the highest frequency (38%) being GGT to GAT. Two cases had mutations at codon 13 (GGT) (glycine) to GAT (aspartic acid)), and one at codon 61 (CAA (glutamine) to CAC (histidine)). An N-*ras* mutation at codon 12 was also detected in one case (GGT (glycine) to GAT (aspartic acid)). A rare insertion-deletion at codon 61 of N-*ras* gene was identified in one Japanese patient. This mutant causes a frameshift to produce a stop codon (TAA) at codon 86. In addition, it was noted that another Japanese patient carried bimutations of the K-*ras* gene at codon 12 (GGT to AGT) and codon 13 (GGT to GAT). However, few mutations in K-*ras* gene have been identified in intrahepatic bile duct cancer among Thai patients. Only 2 out of 52 cases harbor such a mutation, one at codon 12 (GGT to GTT) and one at codon 13 (GGT to GAT).

TUMOR SUPPRESSOR GENES

Tumor suppressor genes are normal cellular genes whose loss of function lead to tumor development. These genes encoded proteins which play a role in negatively regulating the cell cycle. Not only can tumor suppressor genes be inactivated at the gene level but their protein products can also be made nonfunctional by interaction with oncoproteins of DNA viruses (Marshell, 1991). Tumor suppressor genes involved in human cancers include *p16, BARC1, WT1, Rb, APC, NF1, NF2, DCC, MTS1, MST2,* and *p53* (Levine, 1995).

p53 is a tumor suppressor gene frequently found inactivated in a variety of human cancers (Hollstein *et al,* 1991). The human *p53* gene is located on the short arm of chromosome 17 (17p13.1), and spans about 20 kb of genomic DNA, contains 11 exons and encodes a 53 kDa protein.

In normal cell, wild type *p53* protein functions as a suppressor of cell proliferation and inhibits malignant transformation (Soussi *et al,* 1990). *p53* appears to play a crucial role in the cell cycle acting as a check point monitor during transition between G_1 and S phases of the cell cycle (Canman *et al,* 1994). In the event of DNA damage or deregulated growth, wild type *p53* is induced and leads to either cell cycle arrest or programmed cell death (Hooper, 1994). Furthermore, recent findings have revealed that *p53* can directly stimulate DNA repair machinery (Smith *et al,* 1994). Mutation within this gene causes a loss of function, the most common mutation being a single base substitution, although deletion of one or more bases has been detected.

A "hot spot" of mutations is located in exons 5 to 8, between codons 130 to 290 (Hollstein *et al,* 1991). The most common mutations are C to T and G to A transitions at CpG dinucleotides. More than 20 types of human cancer have been reported to carry *p53* gene mutations (Greenblatt *et al,* 1994). Association between *p53* gene mutations and late events in the multistep process of carcinogenesis was demonstrated in colon cancer (Vogelstein *et al,* 1988) and ovarian carcinoma (Teneriello *et al,* 1993). However, in lung cancer *p53* mutation was associated with early stage of carcinogenesis (Sozzi *et al,* 1992).

Study of *p53* mutations in cholangiocarcinoma from Japanese and Thai patients show that the mutations in both ethnic groups are distributed between exons 5 to 8, as summarized in Table 3 (Kiba *et al,* 1993). The most common change is G to A transition which was found in 2 Japanese patients (50% of cases) and in 5 Thai patients (56%). In our study, *p53* gene mutation at codon 282 in exon 8 with C to T transversion resulting in arginine (CGG) to tryptophane (TGG) substitution was detected in only 1 out of 20 Thai patients with cholangiocarcinoma.

CONCLUSION

Mutations in the *ras* genes in cholangiocarcinoma show a broad spectrum. This supports the hypothesis

Table 1

The incidence of *ras* gene mutations in cholangiocarcinoma in various ethnic groups.

Ethnic group (Total no.)	No. of cases with positive mutation[a] K-*ras* codon 12	13	61	N-*ras* codon 12	13	61	References
English[c] (15)	15	-	-	-	-	-	Levi *et al*, 1991
American (3)	2	-	-	-	-	-	Caldas *et al*, 1994
Japanese[c]	1	-	-	-	-	-	Tada *et al*, 1990a
Japanese[d] (30)	13	2	1	1	-	1[b]	Tada *et al*, 1990b; Tada *et al*, 1992; Tsuda *et al*, 1992; Kiba *et al*, 1993;
Thai[d] (32)	1	1	-	-	-	-	Tsuda *et al*, 1992; Kiba *et al*, 1993
Thai[d] (20)	-	-	-	ND	ND	ND	Unpublished data

[a]H-*ras* mutation was not found; [b]insertion-deletion; [c]Extrahepatic bile duct cancer; [d]Intrahepatic bile duct cancer; ND = not determined

Table 2

The spectrum of *ras* gene mutations in cholangiocarcinoma.

Mutant type	Patients English[a] (n =15)	American (n = 3)	Japanese (n = 30)	Thai (n = 52)
K-*ras* codon 12 wild type glycine (GGT)				
Alanine (GCT)	-	-	3	-
Arginine (CGT)	2	-	-	-
Aspartic acid (GAT)	3	2	5	-
Cysteine (TGT)	-	-	2	-
Serine(AGT)	4	-	3	-
Valine (GTT)	6	-	-	1
K-*ras* codon 13 wild type glycine (GGT)				
Aspartic acid (GAT)	-	-	2	1
K-*ras* codon 61 wild type glutamine (CAA)				
Histidine (CAC)	-	-	1	-
N-*ras* codon 12 wild type (GGT)				
Aspartic acid (GAT)	-	-	1	-
N-*ras* codon 61 wild type glutamine (CAA)				
Insertion-deletion	-	-	1	-

[a]1 case = Ser+Arg; 5 cases = Ser+Val; 2 cases = Arg+Asp; 1 case = Ser+Val+Arg

Table 3

p53 mutations in cholangiocarcinoma from Japanese and Thai patients.

Exon	Codon mutation (Amino acid substitution)	Japanese[a] (n = 12)	Thai[a] (n = 26)	Thai[b] (n = 20)
5	154 GGC, glycine to GTC, valine	-	-	-
	175 CGC, aginine to CAC, histidine	-	2	-
	178 CAC, histidine to GAC, aspartic acid	1	-	-
	179 CAT, histidine to CTT, leucin	-	1	-
6	215 AGT, serine to ATT, isoleucine	-	1	-
7	245 GGC, glycine to AGC, serine	1	1	-
	248 CGG, argine to TGG, tryptophan	-	1	-
	256 ACA, thyrosine to AAA, lysine	1	-	-
	258 GAA, glutamine to GGA, glycine	-	2	-
8	273 CGT, arginine to TGT, cysteine	-	1	-
	282 CGG, arginine to TGG, tryptophan	1	-	1

[a]Kiba *et al* (1993); [b]Unpublished data

that *ras* mutation is not the initiating factor in these tumors but occurs during the clonal expansion in a tumor of the initiated cells (Levi *et al,* 1991). There is high frequency of *ras* gene mutations in cholangiocarcinoma from English patients (100%), and Japanese patients (60%) compared with a low frequency in Thai patients (4%). The frequency of *p53* mutations in Thai patients (5%) are lower than in Japanese (33%).

These findings may reflect a different etiology of this cancer among the three groups. English and Japanese patients in these reports were not infected with liver flukes nor had they been exposed to Thorotrast (Tada *et al,* 1990a; 1990b; 1992; Tsuda *et al,* 1992; Kiba *et al,* 1993); whereas all Thai patients were infected with *O. viverrini* (Tsuda *et al, 1992;* Kiba *et al,* 1993).

The frequency of gene mutations in *ras* oncogene and *p53* tumor suppressor gene in Thai patients with cholangiocarcinoma is very low when compared with other types of human cancer (Bos, 1989; Hollstein *et al,* 1991; Greenblatt *et al,* 1994). These results suggest that these genes may not be early targets during bile duct cancer development associated with the liver fluke infection.

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