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Mutational Analysis of K-*ras* and p53 Genes in Human Lung and Pancreatic Carcinoma Cell Lines

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Several types of human lung and pancreatic carcinoma cell lines were cultured and their chromosomal DNAs were extracted. These DNAs were then partially amplified by PCR (Polymerase Chain Reaction) and sequenced to analyze the types and frequency of mutations, and their possible relation in the oncogene, K-*ras* and suppressor gene, p53. Regardless of the cell line origin, 81% were found to possess at least one mutation. Among the cell lines analyzed, 54.5% of the mutations were found in either K-*ras* or p53. Except for one nonsense mutation, all mutations were missense with either base insertions or substitutions. Furthermore, besides the p53 codons Known to be mutated simultaneously with *ras* to enhance tumor growth, p53 164-165 and 248 were found to be mutated simultaneously with K-*ras*. Regardless of the site of p53 mutation, all K-*ras* mutations found in these cases occurred at exon 1, codon 12.

KEY WORDS: Ras, p53, PCR, DNA Sequencing

Ras, known as one of the small GTP-binding proteins like other G proteins conducts signal transduction from the cell-surface receptors to the internal second-messenger systems and whose activity is determined by its bound nucleotide (GTP/GDP) (Gilman, 1987).

Three sequence motifs important for nucleotide interaction which are conserved between guanosine and nucleotide-binding proteins includes, GXXGXGKS (amino acid 10-17), involved in the binding of the α- and β-phosphates, DXXG (57-60), aspartate 57 binds to the Mg^{2+} and glycine 60 to the δ-phosphate when GTP is bound; NKXD (116-119), is important for binding to the guanine ring (Barbacid, 1987; Tada *et al.*, 1990).

Point mutations at residues 12, 13 or 61 have been reported to particularly affect the GTP hydrolysis intrinsic rate (Bollag and McCormick, 1991). Substitution of the normal Gly 12 or Gln 61 by a wide range of amino acids reduces the intrinsic GTPase activity and makes the protein transformable. Residues 32-40 are able to enhance the GTPase actvitiy by five times and hence is known as the effector domain. *ras* mutated within this domain is incapable of transforming back to its inactive state (Bos, 1989; Krengel *et al.*, 1990). Furthermore, when Asp 57 where Mg^{2+} binds is transformed to Thr or when Gly 60 is replaced by Cys or His, abnormal Ras were synthesized (Barbacid, 1987). Mutations at residues 116, 117, 118 or 119 dramatically increase the Ras intrinsic nucleotide exchange rate and since the cellular amount of GTP is greater than GDP, Ras favorably binds to GTP (Gibbs *et al.*, 1990; Bourne *et al.*, 1991; Kalthoff *et al.*, 1993).

In contrast to Ras which functions in the cytoplasm, p53, a tumor suppressor gene located at chromosome 17p3.1 encodes a 53,000 dalton

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nuclear DNA-binding phosphoprotein, consisting of 11 exons in which are three conserved domains. The first highly acidic charged domain (codon 75-80) at the N-terminal is assumed to form an α-helical structure. The second domain between codon 75-150 is a hydrophilic proline rich domain, while the third domain possesses a highly alkaline charged domain at the C-terminal between codon 319-393 which is capable of forming a helix-turn-helix motif (Levine *et al.*, 1991).

The function of p53 is of interest because the gene for p53 is mutated or deleted in 60-70% of human tumors. Induction of wild-type p53 expression in growth-stimulated cells inhibits progression from the G1 phase of the cell cycle to the S phase (Werness *et al.*, 1990).

Two possible functions of p53 are it could regulate the assemble or function of the DNA replication-initiation complex. It may act in the G1 phase of the cell cycle to promote or prevent the assembly of a DNA replication-initiation complex. Alternatively, p53 could act as a transactivator of gene transcription, either promoting or repressing mRNA synthesis (Roauch *et al.*, 1993).

It has been reported that mutations at codon 120-130, 175, 273 and 281 of p53 cooperates with Ras in the cytoplasm to enhance tumor growth (Levine *et al.*, 1991). For p53 to cooperate with Ras, great amount of p53 must be present. This is compensated by the extended half-life of mutated p53 from 6-20 minutes to 2-20 hours and the p53 introns (Hinds *et al.*, 1989). p53 introns are believed to enhance the rate of transcription and the stability of mRNA and hence increase the p53 mRNA content (Hinds *et al.*, 1990).

With the knowledge that the K-*ras* proto-oncogene is involved in signal transduction and that the p53, tumor suppressor gene disrupts the cell cycle, this report aims to study the types, frequency and possible relation of the mutations in several human lung and pancreatic carcinoma cell lines.

Materials and Methods

Cell lines were purchased from ATCC (America Type Culture Collection, Rockville, M.D., USA) and cultured in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal bovine albumin serum and 100 IU/ml pencillin and 100 μg/ml streptomycin in an incubator of 37°C supplied with 5% CO_2.

DNA were extracted from 5×10^7 of each cell lines by GIT buffer (4 M guanidine isothiocyanate, 3M sodium acetate pH 6.0, 0.8% β-mercapthoethanol) and sedimented by 5.7 M cesium chloride. Then repeated phenol/chloroform and ethanol precipitations were performed for purification.

Primers were designed as shown in figure 1. Due to the large conservative sequences in K-*ras*, primers were designed at the introns.

Extracted and purified genomic DNA were amplified by PCR. A PCR cocktail solution of 100 μl containing 1X buffer (500 mM KCl, 100 mM tris-HCl, 1% triton X-100, 25 mM $MgCl_2$), 2.5 mM deoxynucleotide triphosphate, 50 pmole sense and antisense primers, 5 units Taq polymerase and 1-2 μg/ml of extracted DNAs were amplified.

PCR were performed by a thermocycler (Perkin-Elmer Cetus, USA) at an annealing temperature ranging from 53-55°C for 40 cycles.

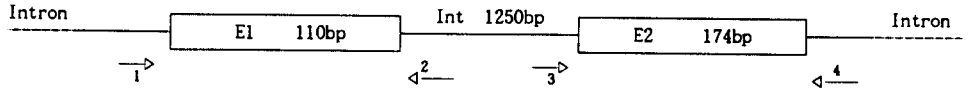
PCR products were observed by gel electrophoresis, purified by repeated phenol/chloroform and ethanol precipitation, and by Gene Clean II Kit (Bio 101).

Sequencing of K-*ras* and p53 were performed by the Sanger Method (Sanger *et al.*, 1977) and sequenced by denaturing gel electrophoresis.

Results and Discussion

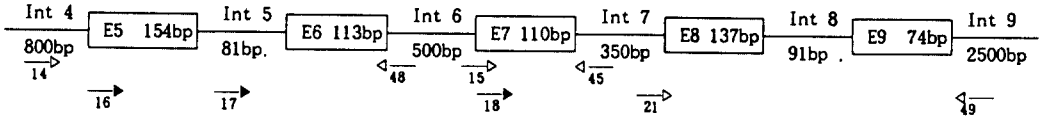
Proto-oncogene *ras* plays an important role in the first part of signal transduction in the cytoplasm while in the nucleus p53 is believed to bind directly to $TGCCT(X)_{5\sim8}$-TGCCT and may play a role as a gene expression transcription activating factor or may regulate the expression of genes that regulate the G1-S phase of the cell

(1) K-*ras*



K-*ras* PCR & Seq. primers : Exon 1 1 ATTATAAGGCCTGCTG
Exon 2 2 GCATATTACTGGTGCAGG
3 TCCAGACTGTGTTTCTCCCT
4 GCTGACATTAAGGAGTAA

(2) p53



p53 PCR & Seq. primers : Exon 5-6 14 ACTTGTGCCCTGACTT
48 AGGGTGGTTGTCAGTAG
Seq. primers : 16 CTGACTTTCAACTCTGTC
17 ATGACGGAGGTTGTGAG
PCR & Seq. primers : Exon 7 15 ACTTGTGCCCTGACTT
45 AATCTACTGGGACGG
Seq. primer : 18 TCATCTTGGGCTGTGTT
PCR & Seq. primers : Exon 8-9 21 GAGCTTAGGCTCCAGAAAG
49 TCAAGTGGAAAGTTTCCA

Fig. 1. Specific primers designed for DNA amplification by PCR (Polymerase Chain Reaction).
Int: Intron —▷ 5′ seq. & PCR primers —▶ 5′ seq. primers
E: Exon ◁— 3′ seq. & PCR primers ◀— 3′ seq. primers
bp: base pairs

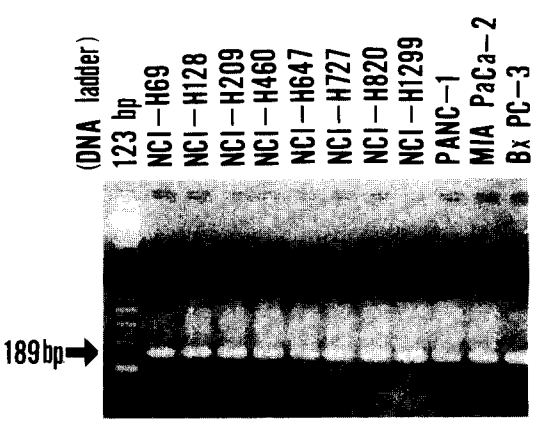


Fig. 2a. Amplification of K-*ras* exon 1 by PCR. PCR products with a size of 189bp were amplified by primers 1 and 2 at an annealing temperature of 55°C.

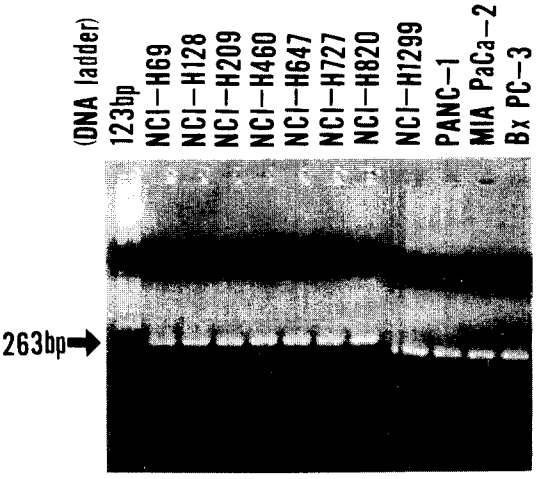


Fig. 2b. Amplification of K-*ras* exon 2 by PCR. PCR products with a size of 263bp were produced by primers 3 and 4 at an annealing temperature of 55°C.

cycle by interacting with the transcription factors. Furthermore, it is also believed to play an important role in DNA replication initiation or during DNA replication complex formation (Fiscella *et al.*, 1993; Bookstein and Allred, 1993).

Studies have shown that in *ras* mutations, 90% were found at K-*ras* while the remaining 10%

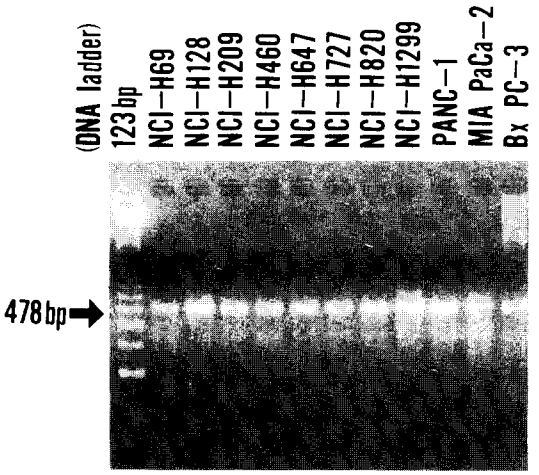


Fig. 3a. Amplification of p53 exon 5 and 6 by PCR. PCR products with a size of 478bp were obtained by primers 14 and 48 at an annealing temperaure of 53°C.

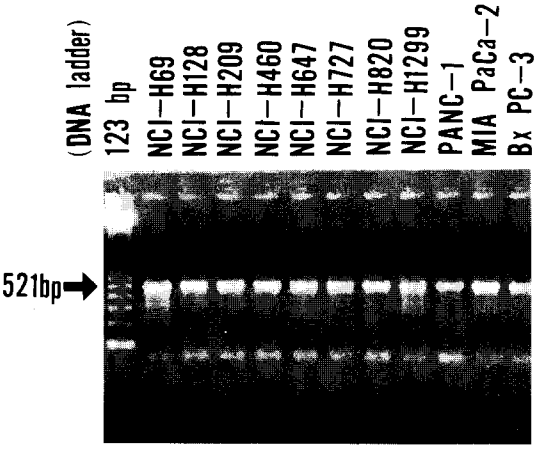


Fig. 3b. Amplification of p53 exon 7 by PCR. PCR products with a size of 521bp were amplified by primers 15 and 45 at an annealing temperature of 53°C.

were found in the H- or N-*ras* (Metcalf *et al.*, 1992). Most mutations were found at codon 12, 13 and 61 (Levine *et al.*, 1991). In p53, mutations mainly occur within exons 5~9 and about 60-70% of the mutations were found in lung cancers (Kern and Filderman, 1993). Hence, in this report, DNA sequencing were performed in K-*ras* exons 1~2 and p53 exons 5~9.

K-*ras* exon 1 (Fig. 2a) was amplified by primers 1 and 2 while exon 2 (Fig. 2b) by primers 3 and 4,

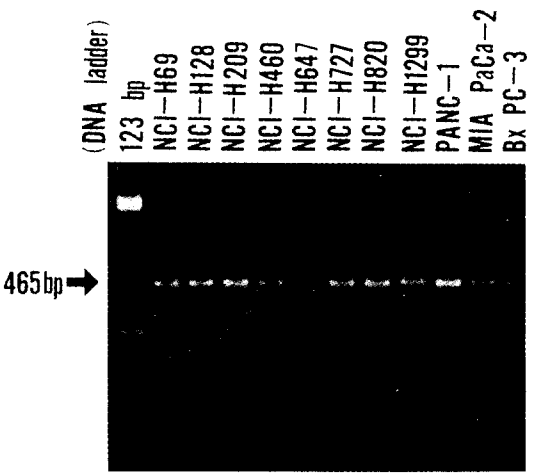


Fig. 3c. Amplification of p53 exon 8 and 9 by PCR. PCR products with a size of 465bp were obtained by primers 21 and 49 at an annealing temperature of 55°C.

at an annealing temperature of 55°C. PCR products of 189 bp and 263 bp were obtained respectively.

p53 exons 5 and 6 (Fig. 3a) were amplified by primers 14 and 48 at an annealing temperature of 53°C producing a PCR product of 478 bp while exon 7 (Fig. 3b) by primers 15 and 45 yielding a product of 521 bp. Exons 8 and 9 (Fig. 3c) were amplified by primers 21 and 49 at an annealing temperature of 55°C producing PCR products of 465 bp.

According to the summary of results shown in Table 1, among the 11 human cancer cell lines studied, 81% possessed at least one mutation at K-*ras* or p53 while 54.5% were found to have either one K-*ras* or p53 mutations.

NCI-H647, NCI-H727, PANC-1 and Bx PC-3 (Fig. 5) were found to have mutations at K-*ras* codon 12(Gly) and 13(Gly) which are believed to code a different amino acid. According to Feig and Cooper (1988) report, it is assumed that these codons do not play a direct role in GTP hydrolysis. Furthermore Pai *et al.*, (1989) observed that when codon 13(Gly) was substituted to Arg or Val, Ras GTP hydrolysis can be inhibited. Base substitution at K-*ras* codon 61(Gln) was observed in NCI-H460 (Fig. 4) and NCI-H1299. This amino acid side chain has been reported to directly interfere

Table 1. Summary of Gene Mutations Found in Human Cell Lines.

No.	Cell Line	Tumor Organ, Cell Type	K-*ras* Mutation Exon/Codon/nt/aa	Type of Mutation	p53 Mutation Exon/Codon/nt/aa	Type of Mutation
1	NC1-H69	Lung, Small Cell Carcinoma			Exon5/Codon171 GAGC→TAG Glu→Stop	Nonsense Mutation (base substitution)
2	NC1-H128	Lung, Small Cell Carcinoma				
3	NC1-H209	Lung, Small Cell Carcinoma				
4	NC1-H460	Lung, Large Cell Carcinoma	Exon2/ Codon61 CAA→CAT Gln→His	Missense Mutation (base substitution)		
5	NC1-H647	Lung, Adenosquamous Carcinoma	Exon1/ Codon13 GGC→GAC Gly→Asp	Missense Mutation (base substitution)		
6	NC1-H727	Lung, Pulmonary Carcinoid	Exon1/ Codon12 GGT→GTT Gly→Val	Missense Mutation (base substitution)	Exon5/ Codon164~165 TAC AAG CAG Tyr Lys Gln	Missense Mutation (base insertion)
7	NC1-H820	Lung, Papillary Adenocarcimoma			Exon8/ Codon284~285 CGC ACA→CGG CCA Arg Thr→Arg Pro	Silent & Missense Mutation (base substitution)
8	NC1-H1299	Lung, Large Cell Carcinoma	Exon2/ Codon 60~61 GGT CAA→GGC AAA Gly Gln→Gly Lys	Silent & Missense Mutation (base substitution)		
9	PANC-1	Pancreatic epitheloid Carcinoma	Exon1/ Codon12 GGT→G(A)GT Gly→Glu	Missense Mutation (base substitution)	Exon8/ Codon273 GCG→GCA Ala→Ala	Silent Mutation (base substitution
10	MIA PaCa-2	Pancreatic Carcinoma	Exon1/ Codon12 GGT→TGT Gly→Cys	Missense Mutation (base substitution)	Exon7/ Codon248 CGG→TGG Arg→Trp	Missense Mutation (base substitution)
11	Bx PC-3	Primary pancreatic Adeno-carcinoma			Exon6/ Codon220 TAT→TGT Tyr→Cys	Missense Mutation (base substitution)

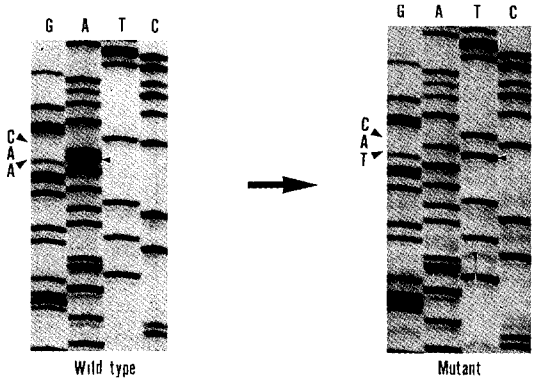


Fig. 4. DNA sequencing result of NCI-H460 K-*ras*. A missense mutation at K-*ras* exon 2, codon 61 occurred with a base substitution from CAA(Gln) →CAT(His).

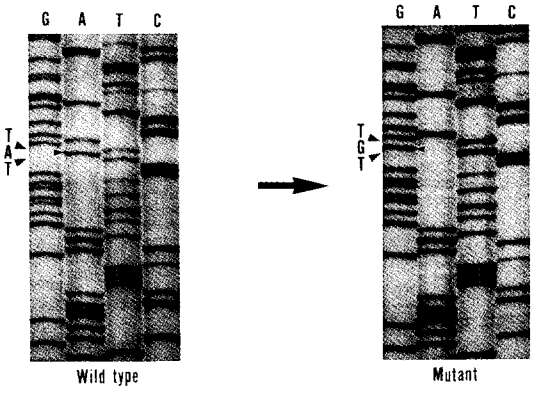


Fig. 5. DNA sequencing result of Bx PC-3 p53. A missense mutation ocurred at p53 exon 6 codon 220 with a base substitution from TAT(Tyr) to TGT(Cys).

in GTP hydorlysis (Zhang *et al.*, 1990)

Among the cell lines observed, 54.5% were found to have p53 mutations and among these, 37.5% were found in lung cancer while 62.5% were found in pancreatic carcinoma cell lines. Hence, it is concluded that p53 is a potent cancer suppressor gene in both lung and pancreatic carcinomas. The p53 mutations found possessed several characteristics: (1) Besides the nonsense mutation found in NCI-H69, all mutations found were missense with base substitution or insertion. DNA sequencing results in NCI-H69 showed a nonsense mutation at p53 exon 5, codon 171 as a base substitution occurred from GAG(Glu) to TAG(Stop). In this case, it is believed that protein translation terminates at codon 171 hence synthesizing an abnormal protein. Base insertion in NCI-H727 with nine bases did not change the reading frame, however one base insertion in PANC-1 is believed to have caused a shift in the reading frame. (2) Mutations found were not dispersed but were scattered within codon 171 and 289 and hence this region may be an important domain for p53 to perform its function.

Mutations in K-*ras* and p53 were found to occur simultaneously in NCI-H727 and MIA PaCa-2. Till recently, p53 codons 120-130, 175, 273 and 281 were found to cooperate with *ras* to enhance tumor growth as *ras* is known to inhibit p53 function to enhance its own activity (Hinds *et al.*, 1990; Hicks *et al.*, 1991). In NCI-H727, p53 exon 5 codon 162, 163 and 164 were repeatedly inserted within codon 164 and 165, while in MIA PaCa-2, a base substitution occurred at p53 exon 7 codon 248. These two cell lines possessed mutations at K-*ras* exon 1 codon 12. As mentioned earlier, codon 12 does not play a direct role in hydrolysis and hence it is believed that mutation at this codon alone is not sufficient to cause cancer. In contrast, mutations found at NCI-H460 and NCI-H1299 at K-*ras* exon 2 codon 61, whose glutamate side chain is known to be directly involved in GTP hydrolysis may be an important site to be able to induce cancer alone.

In PANC-1, a missense mutation occurred at K-*ras* exon 1 codon 12, however a silent mutation occurred at p53 exon 8 codon 273 which is also known to cooperate with *ras* to enhance tumor growth.

No mutations were found in K-*ras* exons 1-2 or p53 exons 5-9 in NCI-H128 and NCI-H209. These cell lines may possess mutations at other exons not studied in this report.

In conclusion, proto-oncogene K-*ras* and tumor suppressor gene p53 have been found to be important cancer causing factors in human lung and pancreas. Furthermore, besides the p53 codons known to cooperate with *ras*, codons 164-165 and 284 have been found to be mutated simultaneously with K-*ras*. Another striking feature is that regardless of the p53 mutations sites, mutations in K-*ras* were both found in exon 1

codon 12 which is known to be indirectly involved in GTP hydrolysis. It is presumed that p53 and K-*ras* may cooperate by a certain regular mechanism to enhance tumor growth. Further research is required to understand the exact mechanism of cooperation between K-*ras* and p53.

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사람 폐암과 췌장암 세포주에서 K-*ras*와 p53 유전자의 돌연변이에 대한 연구

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여러 종류의 폐암과 췌장암 세포주를 배양하여 DNA를 분리하였다. 분리한 DNA는 PCR(Polymerase Chain Reaction)로 증폭하여 염기 서열화를 시행하여 K-*ras*와 p53 유전자들의 돌연변이 종류, 빈도 및 가능한 관계에 대하여 조사하였다. 연구한 암세포주 중 약 81%가 종양 유전자 K-*ras*와 암 억제 유전자 p53 중 적어도 하나의 돌연변이를 가지고 있었으며 두 유전자 각각에 대해서는 암 세포주 중 약 54.5%에서 돌연변이가 나타났다. 발견된 돌연변이의 종류는 1개의 세포주에서 발견된 넌센스 돌연변이 이외에는 모두 미스센스 돌연변이가 일어났으며 2개의 세포주에서 일어난 염기 삽입이외에는 모두 염기 치환이 일어났다. 현재까지 p53 코돈 중 *ras*와 동시에 돌연변이가 일어난다고 보고된 코돈 이외에도 p53 코돈 164-165과 248이 K-*ras*와 동시에 돌연변이가 발생하였고, p53 유전자의 돌연변이 위치에 관계없이 K-*ras* 유전자에서는 exon 1, 코돈 12에서 돌연변이가 발생하였다.