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Absence of a Mutation of the *p21/WAF1* Gene in Human Lung and Pancreatic Cancers

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The expression of a negative regulator of the cell cycle, p21^{WAF1} protein, is trans-activated by wild-type p53, but not by the mutant protein. Therefore, mutations of the *p53* and *WAF1* genes may be complementary. We examined DNAs from 70 human primary lung (63 of NSCLC and 7 of SCLC) and 24 pancreatic cancers (19 primary cancers and 5 cell lines) for mutations of the *WAF1* gene. No mutations were detected in any samples examined, regardless of the mutational state of the *p53* gene. The results suggested that aberrations of the coding sequence of the *WAF1* gene are not associated with carcinogenesis in lung and pancreatic cancers.

Key words: *p21/WAF1* gene — Human lung cancer — Human pancreatic cancer — *p53* gene

The progression of the cell cycle is controlled positively by several CDKs[1)] and negatively by several CDK[5] inhibitors (p15^{MTS2}, p16^{MTS1}, p21^{WAF1}, p27^{Kip1}),[2)] that interact with cyclin-CDK complexes. The protein p21^{WAF1} associates with multiple cyclin-CDK complexes, including cyclin B-cdc2, cyclin D1-CDK4, cyclin E-CDK2 and cyclin A-CDK2, that inhibit cells from entering the S phase.

The *WAF1* gene encoding p21 protein has been identified,[3,4)] and it is trans-activated by wild-type p53, but not by mutant proteins.[3)] Since mice with the *p53* gene knocked out lack detectable *WAF1* expression,[5)] and over-expression of p21 protein inhibits the growth of various types of cancer cells *in vitro*,[3)] p21 protein may be a downstream effector of the p53-mediated suppression of cell proliferation. The p21 protein also controls replication and repair of genomic DNA by interacting with PCNA during the p53-mediated suppression of cell growth.[6)]

We found mutations of the *p53* gene in 52% of human primary NSCLC.[7)] In this study, we examined whether or not abnormalities of the *p53* and *WAF1* genes are complementary in these primary lung and pancreatic cancers. The results suggested that the sequence of the *WAF1* gene is not abnormal, at least in human lung and pancreatic cancers, irrespective of the mutational state of the *p53* gene.

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[5] Abbreviations: CDK, cyclin-dependent kinase; LOH, loss of heterozygosity; NSCLC, non-small cell lung cancer; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; SCLC, small cell lung cancer; SSCP, single-strand conformation polymorphism; WAF, wild-type p53-activated fragment.

MATERIALS AND METHODS

DNA sample Seventy surgical specimens of cancerous and apparently non-cancerous portions of the lung from the same patients were obtained and DNA was extracted as described.[8)] The specimens included 39 adenocarcinomas, 17 squamous cell carcinomas, 6 large cell carcinomas, one adenosquamous cell carcinoma and 7 SCLC. Of these tumors, 24 of 65 had *p53* gene mutations.[7)] Pancreatic cancer specimens were obtained from the Center for Adult Disease, Osaka. They consisted of 13 adenocarcinomas and 6 islet cell carcinomas. Mutations of the *p53* gene were detected in 6 of 13 adenocarcinomas (our unpublished data). DNA samples from 5 pancreatic cancer cell lines (MIA PaCa-2, ASPC-1, PANC-1, BxPC-3, CFPAC-2) were also analyzed.

PCR-SSCP analysis and nucleotide sequence analyses We based the sequences of oligonucleotide primers for PCR on the published cDNA sequence[3,9)] (Table I). The PCR mixture of 5 μl contained 1 μM each of two oligonucleotide primers, 200 μM each of 4dNTPs, 20 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, 100 ng of a DNA template and 0.1 U of TaKaRa taq (Takara, Shiga). The PCR mixture was denatured at 95°C for 5 min, then amplified by 30 cycles, each consisting of denaturation at 95°C for 30 s, renaturation at 55°C for 30 s and elongation at 72°C for 1 min. SSCP[10,11)] was performed by means of electrophoresis in two 5% polyacrylamide gels, with or without 5% glycerol at 40 W for 2 h at 25 and 4°C. The gels were dried and exposed to X-ray films. For sequencing, DNA fragments were eluted from gel pieces corresponding to shifted bands on the SSCP gel and reamplified by PCR as described.[12)] The dsDNA Cycle

Table I. Primers Used in the Amplification of Regions of the *WAF1* Gene and Sequencing

Exon	Codon analyzed	Primers	
		Name	Sequence (5′ to 3′)
2	6–52	E2A-F	AGGCGCCATGTCAGAACCGGCT
		E2A-R	GTGGTGTCTCGGTGACAAAG
	53–118	E2B-F	CGTGAGCGATGGAACTTCGA
		E2B-R	CTCCCCTGAGCGAGGCACAA
	118–141	E2C-F	GGACCTGTCACTGTCTTGTA
		E2C-R	GTCATGCTGGTCTGCCGCCGT
3	155–165	E3-F	AGATTTCTACCACTCCAAAC
		E3-R	GACACAAACTGAGACTAAGGC

Sequencing System (GIBCO BRL, Gaithersburg, CA) was used.

RESULTS

The regions of the *WAF1* gene amplified by PCR are shown in Fig. 1. We analyzed the *WAF1* gene using DNA from 70 primary lung cancers, 14 primary pancreatic tumors and 5 cell lines of pancreatic cancer. As complete nucleotide sequences for introns were not available, regions of the *WAF1* gene analyzed contained 147 of 164 codons in the coding exons of the gene (Fig. 1). The region of exon 2 was amplified by PCR into 3 fragments. As the primers were located in the exon, nucleotide sequence changes in codons 1–5 at the 5′-end of the exon and in codons 142–147 at the 3′-end would be undetectable. The first 7 codons at the 5′-end of exon 3 could not be analyzed.

The results of PCR-SSCP analysis of the E2A fragment are shown in Fig. 2. Two sets of signals due to a polymorphic difference in the nucleotide sequence distinguished two alleles. However, we could not detect any cancer-specific mobility shift in the fragment. We also did not detect any tumor-specific mobility shifts in other DNA fragments analyzed. DNA samples from pancreatic cancers were similarly examined and we did not detect any abnormal mobility shifts of DNA fragments in the SSCP analysis.

We detected three types of mobility shifts, including that shown in Fig. 2, which are due to one unknown and two known polymorphic changes of nucleotide sequences. In Fig. 2, patients LuC63, 164 and 189 were homozygous and carried allele 1, whereas patients LuC138, 167, 191 and 193 carried the homozygous allele 2. Patients LuC116, 185 and 196 were heterozygous and carried both alleles 1 and 2. Nucleotide sequence analysis revealed that the polymorphism was due to a known single base change at codon 31,[13] the Ser codon in allele 1 to an Arg codon in allele 2. Analysis of the DNA samples from 70 individuals revealed that 27 and 34% of them carried homozygous alleles 1 and 2, respectively, while 39% were heterozygous. Among these 19 heterozygous individuals however, we did not find any loss of heterozygosity in their tumor DNAs. In fragment E3, there were two polymorphisms (see Fig. 3A and B). One was due to a known C (allele 1) to T (allele 2) transition at nucleotide position 590. As shown in Fig. 3A, patients

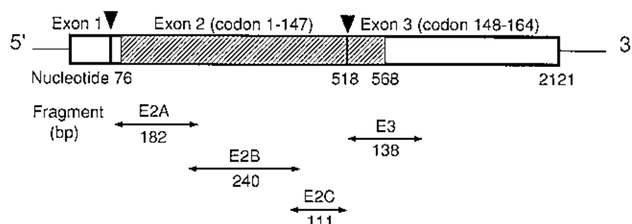

Fig. 1. Regions of the *WAF1* gene amplified by PCR and analyzed by SSCP. The regions of the gene amplified from the genomic DNAs are indicated by bars with arrow-heads. The shaded box indicates the coding region, while the non-coding regions are shown by open boxes. Triangles indicate the exon boundaries.

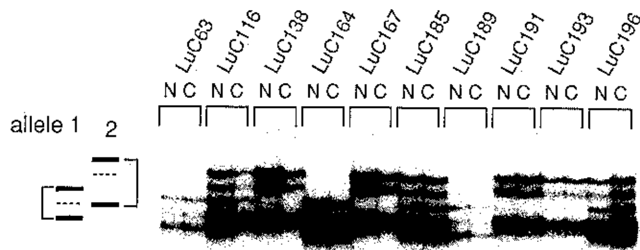

Fig. 2. PCR-SSCP analysis of the region carrying exon 2 of the *WAF1* gene in lung cancers. Pairs of thick bars indicate complementary strands of DNA fragment amplified from alleles 1 (Ser at codon 31) and 2 (Arg at codon 31). Dashed lines indicate signals due to the presence of an isoconformer of the slower-moving strand.

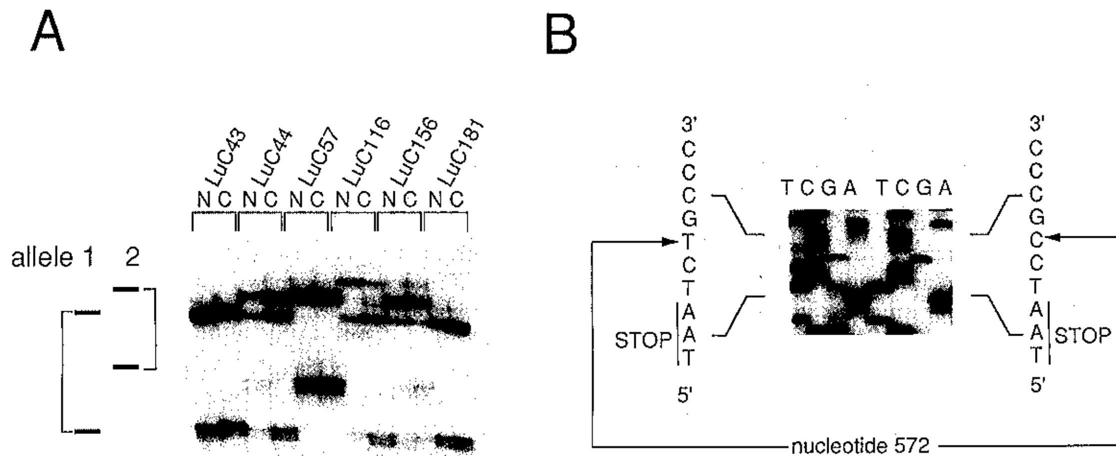

Fig. 3. A polymorphic base change in the non-coding region of exon 3 of the *WAF1* gene. A. Mobility shift due to polymorphisms in SSCP analysis. B. Nucleotide sequence analysis of a DNA fragment carrying a unique polymorphic base change.

LuC43 and LuC181 carried allele 1, while patient LuC57 carried allele 2. Of 70 individuals analyzed, 28 patients including LuC44 and LuC156 (Fig. 3) were heterozygous. However, there was no apparent LOH in the tumors from these informative cases. As shown in Fig. 3, patient LuC116 carried a third allele together with allele 1. Nucleotide sequencing revealed a new polymorphic C-T transition at position 572 in the untranslated region of exon 3 (Fig. 3B). The allele was found in only one of 70 individuals analyzed.

DISCUSSION

As p21^{WAF1} is a downstream effector of p53, the *WAF1* gene could be aberrant in tumors without a *p53* gene abnormality. However, we did not detect any mutations in the regions analyzed for the *WAF1* gene in lung and pancreatic cancers, regardless of the mutational state of the *p53* gene. Furthermore, there was no apparent LOH at the *WAF1* locus (6p21) in informative cases of analyzed tumors, although a putative tumor suppressor gene has been suggested to lie in this region by LOH analysis in some tumors.[14]

Although the precise mechanism for control of expression of the *WAF1* gene has not yet been elucidated, a p53-independent induction pathway of the *WAF1* gene has been identified.[13, 15] Several human growth factors such as fibroblast growth factor, platelet derived growth factor and epidermal growth factor, or some DNA-damaging agents can induce WAF1 expression in cells carrying a mutant *p53* gene as well as in those with the wild-type *p53* gene. Therefore, abnormalities in the upstream regulatory region of the gene which might be complementary to the *p53* gene mutation should be investigated.

Mousses *et al.* reported that the frequency of the variants at codon 31 and nucleotide 590 in tumors with wild-type p53 was significantly greater than in tumors with the p53 mutation.[16] However, in this study, we did not find a significant association of the *WAF1* gene polymorphism at codon 31 (Ser, Arg) with p53 mutational status. This might be due to a difference among the original tissues, since they examined primary breast cancers and sarcomas.

The new polymorphism that we identified in the 3′-untranslated region of exon 3 was infrequent; it was found in only one of 70 patients analyzed.

Our results indicated that an abnormality of the *WAF1* gene, at least in its coding sequence, is not involved in the carcinogenesis of lung and pancreatic cancers. Absence of *WAF1* mutations has also been observed in a variety of human cancers.[17, 18]

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