Advances in Brief

Search for Mutations and Examination of Allelic Expression Imbalance of the *p73* Gene at 1p36.33 in Human Lung Cancers[1]

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Abstract

We examined 61 lung cancer cases to determine whether alterations of p73, a novel monoallelically expressed p53-like molecule, may be involved in the pathogenesis of lung cancer. Allelic loss at the *p73* locus at 1p36.33 was observed in 42% (11 of 26 informative cases), and squamous cell carcinoma tended to carry this lesion most frequently. Somatic mutations in the *p73* gene itself, however, were not detected, despite our extensive search. We found interindividual difference in the allelic expression of *p73* in normal lung, as well as intertissue variance, even within the same individual, but preferential loss of the expressed allele appeared to be an unlikely mechanism for *p73* inactivation. This study, consequently, suggests the presence of an as yet unidentified tumor suppressor gene or genes within the subtelomeric region of 1p, warranting further studies aimed at its isolation.

Introduction

Allelic losses are hallmarks of chromosomal regions harboring tumor suppressor genes. Cytogenetic and molecular genetic studies have demonstrated frequent chromosomal deletions in lung cancer on 3p, 5q, 8p, 9p, 11p, 13q, 17p, 18q, and 22q, suggesting the presence of tumor suppressor genes in the affected chromosomal regions (1). Indeed, we and others have reported genetic alterations of candidate tumor suppressor genes in lung cancer, which include *p16* on 9p, *Rb* on 13q, and *p53* on 17p, as well as *Smad2* and *Smad4/DPC4* on 18q (2–8). Among these genetic lesions, inactivation of *p53* appears to be the most frequent, suggesting an important role of this gene in the pathogenesis of lung cancer.

Recently, Kaghad *et al.* (9) reported a novel gene encoding a protein, which they termed p73, with remarkable sequence similarity to the DNA-binding, transactivation, and oligomerization domains of p53. It was shown that p73 could activate transcription of p53-responsive genes and inhibit cell growth in a p53-like manner, by inducing apoptosis (10). Interestingly, the *p73* gene was mapped to the 1p36.33 region, which is known to be frequently deleted in neuroblastoma, as well as in other types of human cancers, including malignant melanoma, hepatocellular carcinoma, and breast cancer (11–16). In addition, they found that *p73* was monoallelically expressed in neuroblastoma cell lines, as well as in peripheral blood cells of normal donors (9). These observations are particularly of note, in that it has been suggested that a putative tumor suppressor at 1p36 in neuroblastoma is paternally imprinted and maternally expressed, based on the finding of significant maternal bias with regard to origins of discrete 1p36 deletions in neuroblastoma cases.

Here, we investigated the potential involvement of *p73* alterations in the pathogenesis of lung cancer by examining 61 lung cancer specimens for the presence of allelic loss at the *p73* locus and for mutations and allelic expression imbalance of the *p73* gene, with reference to the *p53* gene status in each of lung cancer cases.

Materials and Methods

Tissue Specimens. Tumor samples, along with uninvolved lung tissue where available, were collected from 61 patients diagnosed histologically as having lung cancers (4 cases of SCLC,[3] 26 cases of adenocarcinoma, 28 cases of squamous cell carcinoma, and 3 cases of large cell carcinoma). All tissues were quickly frozen in liquid nitrogen and stored at −80°C until analyzed. In addition, normal lung specimens obtained from three patients undergoing thoracic surgery due to noncancerous diseases, as well as the peripheral blood cells of their parents, were also examined.

Examination of Allelic Loss of the *p73* Locus by PCR-SSCP Analysis. PCR-SSCP analysis was used to distinguish two distinct alleles, representing tightly linked polymorphisms in exon 2 (9). The primer pair used was: GS1 (sense; 5′-CAGGCCCACTTGCCTGCC) and GAS1 (antisense; 5′-AGAGGT-GCTCAAACGTGG). PCR amplification was carried out using genomic DNAs in the presence of [^{32}P]dCTP, followed by electrophoretic separation on 6% nondenaturing polyacrylamide gels with 5% glycerol at room temperature. The PCR amplification consisted of 35 cycles (94°C for 30 s, 60°C for 30 s, and 72°C for 30 s) after the initial denaturation step (94°C for 5 min). Tumor specimens were scored as having allelic loss when the decrease in signal intensity seen in the densitometric tracing was >50%.

Southern Blot Analysis. Southern blot analysis was carried out by using *Eco*RI-digested DNAs. A cDNA probe, which covered the entire open reading frame of the *p73* gene, was prepared by PCR amplification using the following three sets of oligonucleotide primer pairs: S1 (sense; 5′-GGCTGCGACGGCT-GCAGA) and AS3 (antisense; 5′-GCTCATAGGGCACCACGA); S4 (sense; 5′-GTGGATGACCCTGTCACC) and AS6 (antisense; 5′-GGCTGGGGTCG-GCGTGGTA); and S7 (sense; 5′-TCTCGGGGTCCCACTGCA) and AS8 (antisense; 5′-GAGGCAGCTTGGGTCTCT). The PCR amplification consisted of 35 cycles (94°C for 1 min, 60°C for 45 s, and 72°C for 1 min) after the initial denaturation step (94°C for 5 min).

RT-PCR-SSCP Analysis. PCR amplification using random primed cDNAs was performed with the aid of oligonucleotide primers in the presence of [^{32}P]dCTP, followed by electrophoretic separation on 6% nondenaturating polyacrylamide gels, both in the presence of 5% glycerol at room temperature and in the absence of glycerol at 4°C. The primer pairs used were: S1 (sense; see above) and AS1 (antisense; 5′-CGCGGCTGCTCATCTGGT); S2 (sense; 5′-ATCTGCTGAGCAGCACCA) and AS2 (antisense; 5′-TGGCGATCTG-GCAGTAGA); S3 (sense; 5′-ACTCCCCGCTCTTGAAGA) and AS3 (antisense; see above); S4 (sense; see above) and AS4 (antisense; 5′-CGTTCTT-GGCGGAGCTCT); S5 (sense; 5′-GGAGCAGCAGGCCCTGAA) and AS5 (antisense; 5′-CGTAGGACGGGGGCTGTA); S6 (sense; 5′-GCTCCTACA-

[3] The abbreviations used are: SCLC, small cell lung cancer; SSCP, single-strand conformation polymorphism; RT-PCR, reverse transcription-PCR.

Histology	Number examined[a]	p73		p53 mutation[c]
		Mutation	Allelic loss[b]	
Non-small cell lung cancer				
Squamous cell carcinoma	17 (28)	0	6 (10)	9
Adenocarcinoma	21 (26)	0	3 (13)	7
Large cell carcinoma	2 (3)	0	1 (1)	1
SCLC	4 (4)	0	1 (2)	3

[a] Numbers in parentheses indicate numbers of cases examined for the presence of allelic loss at the p73 locus.

[b] Numbers in parentheses indicate numbers of informative cases.

[c] Compilation of data from Suzuki, *et al.* (22), Takagi, *et al.* (23), and unpublished observations.[4]

GAGGCCGAG) and AS6 (antisense; see above); S7 (sense; see above) and AS7 (antisense; 5'-CTGAGCCGCCGATGGAGA); and S8 (sense; 5'-TGCTCCGCTCTAGCAACG) and AS8 (antisense; see above). The PCR amplification in the presence (S1-AS1, S5-AS5, S6-AS6, and S7-AS7) or absence (S2-AS2, S3-AS3, S4-AS4, and S8-AS8) of 10% glycerol consisted of 35 cycles (94°C for 45 s, 60°C for 30 s, and 72°C for 1 min) after the initial denaturation step (94°C for 5 min).

RT-PCR products of lung cancer specimens showing distinct PCR-SSCP patterns were cloned into the *Eco*RV site of pBluescript SKII(−) (Stratagene) after polishing, and the resulting plasmid DNAs prepared from pooled clones were sequenced. RT-PCR products of the corresponding normal lung RNAs were also subjected to PCR-SSCP and sequencing analysis.

Allelic Expression Imbalance Analysis. Allelic expression imbalance was examined by using polymorphisms within the 5'-untranslated region in exon 2 and by means of RT-PCR-SSCP with S1 (sense) and AS1 (antisense) oligonucleotide primers in the presence of [³²P]dCTP. Conditions for PCR amplification and electrophoresis were the same as described above.

Results and Discussion

Sixty-one lung cancer cases were examined for the presence of allelic loss at the p73 locus by PCR-SSCP analysis, as a result of which two distinct alleles representing tightly linked polymorphisms in exon 2 could be distinguished. Among the 26 informative cases, allelic loss at the p73 locus was observed in 11 (42.3%; 1 of 2 SCLCs, 3 of 13 adenocarcinomas, 6 of 10 squamous cell carcinomas, and 1 of 1 large cell carcinoma; Table 1 and Fig. 1). Allelic loss at the p73 locus was observed more frequently in squamous cell carcinoma (60%, 6 of 10 informative cases) than it was in adenocarcinoma (23%, 3 of 13 informative cases).

Identification of frequent allelic loss at the p73 locus, especially in squamous cell carcinomas, led us to investigate whether the p73 gene itself is altered as a target for the frequent allelic loss at 1p36.33, where p73 resides. Forty-four lung cancer specimens with known p53 status were chosen for this purpose and were further examined. Southern blot analysis was performed first to detect gross genomic alterations but failed to identify any such abnormalities (data not shown). RT-PCR-SSCP analysis was then performed to search for subtle mutations in the p73 gene. Distinct mobility shifts were observed with the aid of S5-AS5 and S7-AS7 primer pairs (Fig. 2), as well as the S8-AS8 PCR primer set, in both lung cancer specimens and the corresponding normal lung tissues. Subsequent sequence analysis revealed that these mobility shifts were due to silent nucleotide substitutions at codon 336 (GCC to GCT), codon 349 (CAT to CAC), codon 557 (GCG to GCA), and codon 610 (GCG to GCA), indicating that they probably reflect sequence polymorphisms among individuals. We noted that these sequence variations were concurrently present in all of the eight cases with distinct mobility shifts, whereas two heterozygous cases showing allelic loss exhibited no

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[4] N. Haruki, S. Nomoto, H. Konishi, T. Takahashi, and T. Takahashi, unpublished observations.

mobility shifts for any of the polymorphisms in the lung cancer specimens (Figs. 1 and 2, case 39; case 30, data not shown). There was no such linkage between these silent polymorphisms in the coding exons and the sequence variations in noncoding exon 2. Although p73 mutations could eventually be identified by means of selective screening of a larger number of cases with 1p loss and absence of p53 mutations, the results nevertheless indicated that
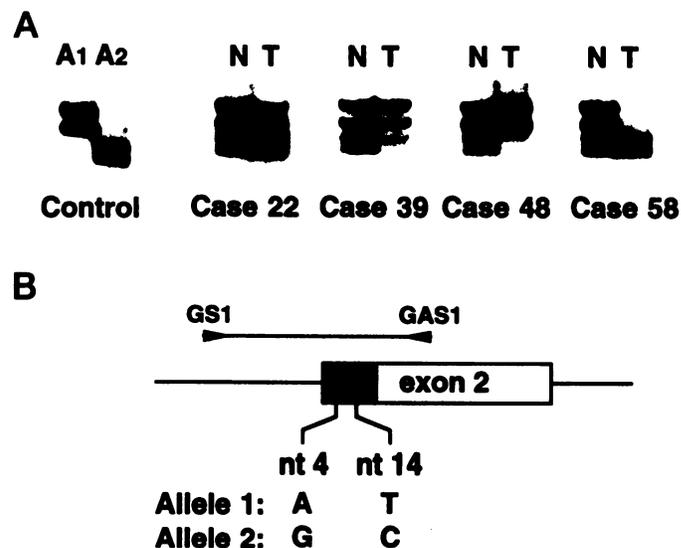

Fig. 1. Allelic loss at the p73 locus at 1p36.33 in representative lung cancer specimens. A, demonstration of allelic loss at the p73 locus by PCR-SSCP analysis using genomic DNAs from lung tumor (*Lanes T*) and normal (*Lanes N*) tissues. *Lane A1*, homozygote carrying A and T at positions 4 and 14 of exon 2; *Lane A2*, homozygote with G and C at these positions. B, schematic illustration of PCR-SSCP analysis for the detection of allelic loss at the p73 locus.
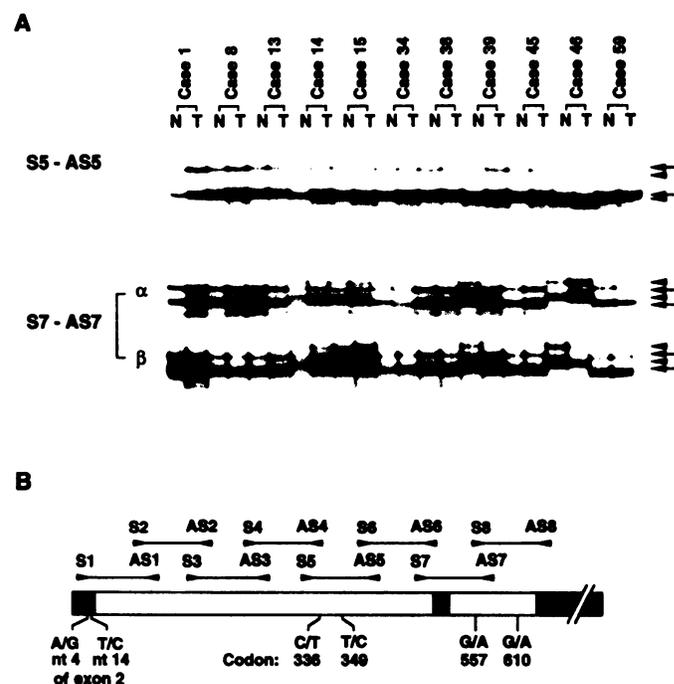

Fig. 2. RT-PCR-SSCP analysis of the p73 gene in lung cancer specimens. A, representative results of RT-PCR-SSCP analysis using the S5-AS5 and S7-AS7 primer sets. Distinct mobility shifts are seen in both normal (*Lanes N*) and tumor (*Lanes T*) tissues of cases 14, 15, and 46, as well as in the normal lung of case 39. α and β, p73α and p73β mRNA isoforms, respectively. B, schematic illustration of the identified silent sequence variations and the strategy for RT-PCR-SSCP analysis of p73, as well as location of the PCR primers. □, coding region; ■, 5'- and 3'-untranslated regions.

mutations in the *p73* gene itself are rare in lung cancer, if present at all. Thus, the conventional two-hit theory did not appear to be applicable as a major inactivation mechanism leading to loss of function of this p53-like molecule in lung cancer.

Genomic imprinting has been suggested as playing a role in certain pediatric tumors, such as Wilms' tumor, based on the findings of highly selective loss of maternal alleles in these tumors (17, 18). Although neuroblastomas had previously been reported to exhibit selective maternal loss of 1p36 (11), Kaghad *et al.* (9) found that the *p73* gene was monoallelically expressed in peripheral blood cells of all five of their cases and in a single neuroblastoma line, lending support to the notion that *p73* might be a candidate tumor suppressor gene in this childhood tumor. We previously reported the identification of genomic imprinting of a cyclin-dependent kinase inhibitor, $p57^{KIP2}$, at 11p15 and selective loss of the expressed maternal allele in lung cancer specimens, suggesting that $p57^{KIP2}$ may be a target for frequent 11p15 deletions in lung cancer (4). We, therefore, examined whether the *p73* gene is also monoallelically expressed in the lung and whether the frequent allelic loss at the *p73* locus observed in this study occurs selectively on the expressed allele of p73. Among 61 lung cancer cases examined in this study, 26 proved to be useful for this study because of their heterozygosities for the polymorphism within its 5'-untranslated region. In contrast to the report by Kaghad *et al.* (9), we observed biallelic expression in the majority (25 of 26) of the normal lungs, although slight variations in certain specimens, such as cases 39 and 42, were identified in this analysis. Although marked allelic expression imbalance was observed only in a single case (Fig. 3, case 48), we had fortunately collected various normal tissues from this patient at necropsy. Examination of the allelic expression status of *p73* revealed significant variations among various normal tissues taken from this individual. The *p73* gene was preferentially expressed from the *A1* allele in lung and liver, whereas expression from the *A2* allele was predominant in the stomach. Small intestine, spleen, and kidney exhibited almost equal expression of the *p73* alleles. These findings indicated that allelic expression of the *p73* gene in the lung varies among individuals and that there is also considerable intertissue variation, even within the same individual.

Marked allelic expression imbalance was also observed in an additional case among three normal lung specimens collected from patients with noncancerous diseases (data not shown). It is of note that the expressed allele in this particular case could be determined to have been paternally derived, in contrast to the findings of Kaghad *et al.* (9), in which the expressed allele in peripheral blood cells was of maternal origin. Because parental origins of the expressed allele of *p73* have been determined only in one case each by us and Kaghad *et al.* (9), further studies are obviously necessary to determine whether *p73* is expressed from a specific parental allele in a tissue-specific manner or whether it is monoallelically expressed regardless of the parental origin. In this regard, it is interesting to note that Mitsuya *et al.* (19) recently demonstrated the presence of either paternal or biallelic expression of *WT1* in human fibroblasts and lymphocytes, in contrast to the previous findings concerning its maternal or biallelic expression in human placental villus and brain tissue (20). It is, therefore, possible that a tissue- and individual-specific modifier(s) might be present and might determine the allelic expression pattern, resulting in a variable allele-specific expression pattern of certain genes, such as *p73* and *WT1*.

Here, we have shown that it appears that the *p73* gene quite infrequently carries mutations in lung cancer, if they occur at all. Because marked allelic expression imbalance in the lung was observed in a very small fraction of the cases examined, it appears to be unlikely that preferential loss of the expressed allele serves as an important inactivating mechanism. We, thus, conclude that the *p73*
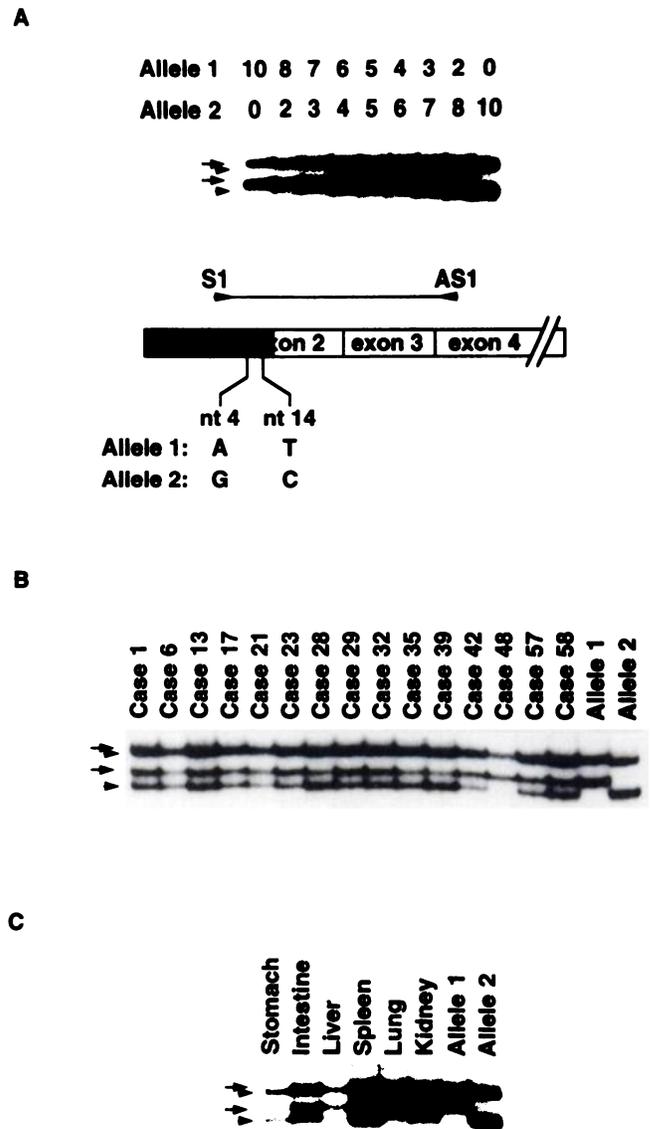

Fig. 3. RT-PCR-SSCP analysis for the detection of allelic expression imbalance of the *p73* gene in normal lung. *A*, mixing experiments using cases homozygous for either the *A1* or *A2* allele of the double nucleotide substitutions in exon 2. *B*, representative results of RT-PCR-SSCP analysis using RNAs of normal lung specimens heterozygous for the *A1* and *A2* alleles. The majority of the cases shows biallelic expression, but case 48 exhibits preferential expression of the *A1* allele. *C*, RT-PCR-SSCP analysis using RNAs from various tissues taken from case 48. Significant variation in allelic expression status is seen among various tissues.

gene is unlikely to play a major role in the pathogenesis of lung cancer. However, the present demonstration of highly frequent allelic loss at the *p73* locus at 1p36.33 suggests that there may be a tumor suppressor gene or genes within this subtelomeric region of chromosome 1p, which may be involved specifically in the development of squamous cell carcinoma. The presence of frequent allelic loss at 1p has also been described in various other tumor types, including neuroblastoma, breast cancer, melanoma, and hepatocellular carcinoma (11–16). Although such frequent involvement of 1p in lung cancer has not yet been reported (21), our use of the subtelomeric marker *p73* in this study seemed to have enabled us to identify frequent allelic loss. An obvious next step toward positional cloning of this putative tumor suppressor gene will be to narrow down its potential location, using increasingly available, closely spaced microsatellite markers. Future studies should lead to the isolation and

characterization of the target gene(s), ultimately leading to a better understanding of the molecular pathogenesis of this fatal disease.

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