[CANCER RESEARCH 58, 2347–2349, June 1, 1998]

Advances in Brief

Activation of *p73* Silent Allele in Lung Cancer[1]

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Abstract

p73, a first p53 relative, has recently been identified and demonstrated to be monoallelically expressed. This protein shows significant amino acid sequence and functional similarities to p53. However, it is unclear whether this protein functions as a tumor suppressor. To elucidate the role of p73 in tumor development, we investigated the expression of the *p73* gene in lung cancer. In a comparison between normal lung and tumor tissues, *p73* was more highly expressed in tumors. Moreover, using a C/T polymorphism in exon 2 for allele-specific expression analysis in 21 pairs of lung tumors and matched normal tissues, we found that five heterozygous samples exclusively expressed both alleles in tumors while showing monoallelic expression in matched normal tissues. This result was confirmed by single-nucleotide primer extension analysis. Mutation analysis of all 13 coding exons of the gene in 21 lung tumor DNAs revealed several polymorphisms, but no tumor-specific mutations were detected. These findings strongly suggest that p73 may play an important role in lung tumorigenesis through activation of a silent allele and overexpression of wild-type p73 rather than as a tumor suppressor.

Introduction

The protein p53 is the most frequently mutated tumor suppressor gene to be identified thus far in human cancers (1, 2). The ability of p53 to inhibit cell growth is due to its ability to bind to specific DNA sequences and activate the transcription of target genes, including the cell-cycle inhibitor $p21^{Waf1/Cip1}$ (3). Loss or inactivation of p53 is thought to contribute to the development of 50% of all human cancers (1, 2). A gene has recently been identified that is predicted to encode a protein with significant amino acid sequence similarity to p53 (4, 5). Each of the p53 amino acid residues that are implicated in direct sequence-specific DNA binding is conserved in this protein (6, 7). This gene, called *p73*, maps to the short arm of chromosome 1 and is found in a region that is frequently deleted in neuroblastomas (4). Jost *et al.* (8) recently demonstrated that p73 can, when overproduced, activate the transcription of p53-responsive genes and inhibit cell growth in a p53-like manner by inducing apoptosis. The structural similarities, its provocative chromosomal location, and the finding that both stimulate p21 expression suggest that both proteins might have similar roles in the cell.

Although p73 has a striking similarity to p53, there are some significant differences between these two proteins.: (*a*) *p73* is a monoallelically expressed gene; only one allele is exclusively expressed in all of the cell lines and normal individuals examined (4); (*b*) *p73* is not inducible by exposure of cells to DNA-damaging agents such as UV irradiation (4); and (*c*) the two proteins are the most divergent in their transcription activation domains, suggesting that p73 could be regulated differently from p53.

To investigate how p73 is involved in tumorigenesis, we focused on lung cancer, because it is the most common human cancer in the United States and other industrial countries (9). It arises as the result of multiple hits that involve amplifications of different oncogenes (10) and the accumulation of mutations in tumor suppressor genes (11). Previous work has also demonstrated that p53 has a higher mutation frequency in lung cancer (70%) than in any other cancers examined (12). We therefore examined p73 in lung tumor specimens. We examined the level of expression of p73 in the lung tumors compared to normal controls and also examined this gene for mutations. We also used a polymorphism present in this gene to examine the expression of the two *p73* alleles in lung tumors, as compared to the monoallelic expression of *p73* in normal lung.

Materials and Methods

Samples. Tumor samples and uninvolved lung tissues were collected at the Mayo Clinic (Rochester, MN). All of the samples were obtained by surgery and were then quickly frozen in liquid nitrogen or transferred to a −80°C freezer. DNA and RNA were extracted with Easy-DNA Kits (Invitrogen) and Trizol RNA extraction solution (Life Technologies, Inc.), respectively.

Semiquantitative RT[3]-PCR. RT was performed with 5 μg of total RNA and Superscript II (Life Technologies, Inc.). *p73* RT-PCR primers are 5′-AACGCTGCCCCAACCACGAG-3′ and 5′-GCCGGTTCATGCCCCCTACA-3′. β-Actin primers are 5′-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3′ and 5′-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3′. PCR was performed using AmpliTaq Gold and 9600 PCR thermal cycler (Perkin-Elmer Corp.) under the following conditions: 95°C for 9 min for activation of AmpliTaq, followed by 95°C for 20 s, 60°C for 15 s (64°C for β-actin), and 72°C for 30 s for 25 cycles with [α-^{32}P]dCTP incorporation.

Allelic Expression of p73. To identify heterozygous samples, intronic primers P1 and P2 (5′-CAGGAGGACAGAGCACGAG-3′ and 5′-CGAAGGTGGCTGAGGCTAG-3′, respectively) were used to perform PCR on DNA isolated from uninvolved lung tissue obtained from 25 patients. Using the above PCR conditions and 35 cycles, a specific 229-bp fragment was obtained. Five μl of the PCR product were then digested with *Sty*I overnight. The heterozygous samples were chosen for RT-PCR with primers P3 and P4 (5′-GGGCTGCGACGGCTGCAGAGC-3′ and 5′-GAGAGCTCCAGAGGTGCTC-3′, respectively) using the same PCR conditions, except that the amplifications were performed twice (25 cycles each). Five μl of the RT-PCR product were digested with *Sty*I overnight. The digestion results were analyzed on an 8% acrylamide gel.

SNuPE. The SNuPE method is as previously described (13). Ten ng of PCR products and 5 pmol of primer (5′-CTTCCCCACGCCGGCCTCC-3′) were used in each sample with [α-^{32}P]dATP for the AT allele and [α-^{32}P]dGTP for the GC allele.

DHPLC Analysis and Direct Sequencing. DHPLC analysis was carried out on an automated DHPLC instrument (Varian) with a column containing alkylated nonporous polystyrene divinylbenzene particles (Sarasep, Inc). Crude PCR products (4–7 μl) containing approximately 50–100 ng DNA were denatured for 3 min at 95°C and followed by gradual reannealing from 95°C

Received 2/6/98; accepted 4/16/98.

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[1] This work was supported by the Mayo Foundation, by NIH Grant CA 48031 (to D. I. S.), and National Cancer Institute Grant CA77118 (to P. Y.).

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[3] The abbreviations used are: RT, reverse transcription; DHPLC, denaturing high-performance liquid chromatography; SNuPE, single-nucleotide primer extension; LOI, loss of imprinting.

to 65°C over a period of 30 min prior to analysis. The heteroduplexes and the homoduplexes were distinguished as the treated PCR products were eluted with an acetonitrile linear gradient in about 4–7 min at 60–65°C based on the GC content of each exon analyzed. PCR products that showed heteroduplex by DHPLC analysis were cleaned with exonuclease and shrimp alkaline phosphatase based on the protocol provided by United States Biochemical and sequenced with fluorescent terminators on an ABI Prism 377 Sequencer (Perkin-Elmer).

Results and Discussion

We first compared *p73* expression in tumor and matched normal samples by semiquantitative RT-PCR with β-actin as a control. Increased *p73* expression was evident in 9 of the 10 tumor samples tested (Fig. 1). The higher expression of *p73* in tumor samples may be due to modifications in the *p73* promoter or may be due to epigenetic modification of *p73* genomic DNA during lung tumorigenesis. This could also result from activation of the *p73* silent allele, given that *p73* was previously demonstrated to be monoallelically expressed.

To test whether the difference in tumor *vs* normal tissue of *p73* expression was due to allelic activation, we performed biallelic expression analysis using the C/T polymorphism in exon 2 of the *p73* gene. Five heterozygous individuals were identified in a total of 21 samples tested by genomic PCR amplification using primers located in introns 1 and 2, followed by *Sty*I digestion (Fig. 2, *a* and *b*). Allelic expression patterns of *p73* in the paired tumor and normal lung tissue samples from these informative individuals were analyzed using cDNA primers that flank the C/T polymorphism (Fig. 2, *a* and *c*). For all five individuals, monoallelic expression was identified in the normal lung samples. In the corresponding five tumor samples, however, both alleles were expressed. Interestingly, the only allele expressed in normal lung tissues from the five informative individuals was the G/C allele. Also, 20 of 21 patients with lung cancers only expressed the G/C allele in their normal tissues (Table 1). Why most of the normal lung tissues exclusively express the G/C allele is now under further investigation.

Because the sensitivity of the RT-PCR method to determine monoallelic *versus* biallelic expression is limited, we performed the SNuPE assay (13). The results of this analysis are consistent with the data obtained by RT-PCR and show that normal tissues only extend dGTP (Fig. 2*d*), indicating that in those samples only the G/C allele is expressed. However, in the five corresponding tumor samples, both

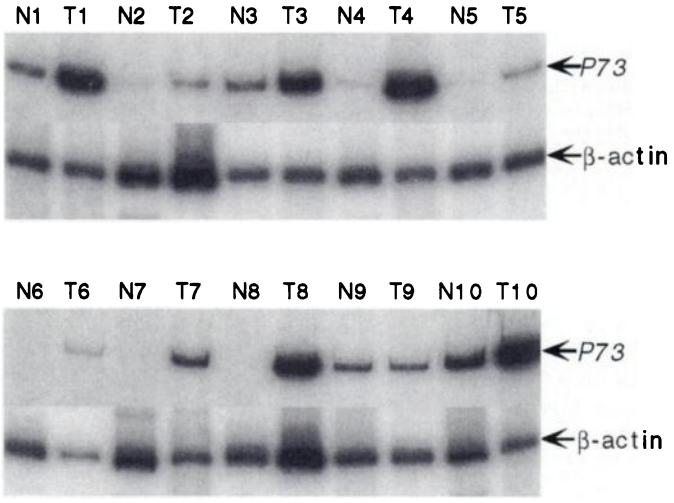

Fig. 1. Comparison of p73 expression between lung tumor and normal lung tissues. Ten pairs of lung tumor (*T*) and matched normal lung tissue samples (*N*) were analyzed. Equal amounts of RNA were used for both *p73* and β-actin amplification. Higher p73 expression in tumors than in their matched normal samples are shown in T1–T8 and T10.

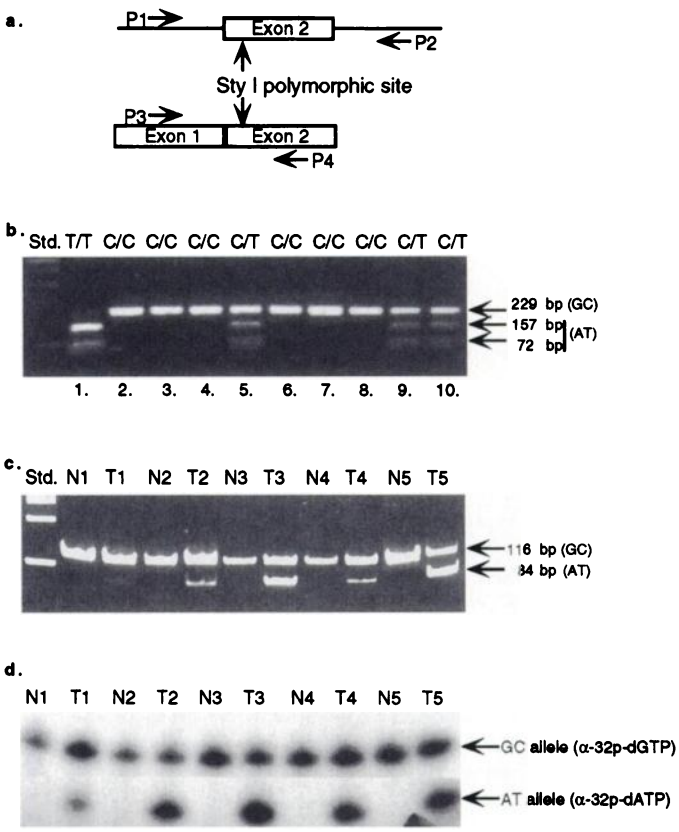

Fig. 2. Analysis of allelic expression of *p73* in lung cancers. *a*, schematic diagram of 5′ *p73*, indicating the location of the *Sty*I polymorphic site in the *p73* gene and the primers used for genomic PCR (*P1* and *P2*) and for RT-PCR (*P3* and *P4*). *b*, representative results showing three samples heterozygous for the *Sty*I polymorphism (*Lanes 5, 9,* and *10*) detected by agarose gel electrophoresis of *Sty*I-digested genomic PCR products from 10 normal lung samples. *Std.*, standard. *c*, biallelic expression of p73 in five informative pairs of lung tumors (*T*) and matched normal lung tissues (*N*). All of the tumor samples showed biallelic expression of the *p73* gene, but their matched lung normal tissues only expressed the gene monoallelically. *Std.*, standard. *d*, SNuPE analysis of allelic expression of *p73* gene in five informative individuals; *top gel*, incorporation of dGTP, which only extends in the presence of the GC allele; *bottom gel*, incorporation of dATP, which only extends in the presence of the AT allele.

Table 1 *Allelic expression of p73 in 21 pairs of lung tumor and normal tissues*

Case	Histology[a]		*Sty*I RFLP[b]	Expressed allele(s)[c]		Overexpressed[d]
	Tumor type	Grade	DNA	Normal	Tumor	in tumors
1	aca	3	G/C; G/C			Yes (T1)
2	sq	3	G/C; A/T	G/C	G/C; A/T	Yes (T2)
3	aca	4	G/C; G/C			Yes (T3)
4	aca	3	G/C; G/C			Yes (T4)
5	sq	4	G/C; G/C			Yes (T5)
6	aca	2	G/C; G/C			ND
7	aca	2	G/C; G/C			ND
8	sq	3	G/C; G/C			Yes (T6)
9	aca	3	G/C; A/T	G/C	G/C; A/T	Yes (T7)
10	sq	3	G/C; G/C			ND
11	sq	3	G/C; A/T	G/C	G/C; A/T	Yes (T8)
12	aca	2	G/C; G/C			No (T9)
13	sq	3	G/C; G/C			Yes (T10)
14	sq	4	G/C; G/C			ND
15	aca	3	A/T; A/T			ND
16	aca	3	G/C; G/C			ND
17	sq	3	G/C; G/C			ND
18	aca	3	G/C; A/T	G/C	G/C; A/T	ND
19	aca	3	G/C; A/T	G/C	G/C; A/T	ND
20	aca	3	G/C; G/C			ND
21	aca	2	G/C; G/C			ND

[a] aca, adenocarcinoma; sq, squamous cell carcinoma.
[b] RFLP detected by *Sty*I restriction exzyme digestion.
[c] The five pairs of tumor and matched normal lung samples listed here (cases 2, 9, 11, 18, and 19) are the N1/T1, N2/T2, N3/T3, N4/T4, and N5/T5 respectively, shown in Fig. 2*c*.
[d] T1–T10 listed here are the same 10 tumor samples used for expression analysis with their matched normal lung tissue samples in Fig. 1. ND, not determined.

Table 2 *Polymorphisms identified in the p73 gene*

Nucleotide change[a]	Amino acid change	Exon/Intron	Heterozygosity[b]
G81A	None	Exon 2	0.24
C91T	None	Exon 2	0.24
T629C	None	Exon 5	0.24
C1118T	None	Exon 9	0.1
T1157C	None	Exon 9	0.1
G1940A	None	Exon 14	0.05
Exon 6 + 52 (G/C)		Intron 6	0.38
Exon 8 + 15 (G/A)		Intron 8	0.43
Exon 8 + 40 (G/A)		Intron 8	0.43
Exon 13 − 65 (G/A)		Intron 12	0.29

[a] Nucleotide position is based on the cDNA sequence in the European Molecular Biology Laboratory database under the accession no. Y11416 EMBL.

[b] Polymorphisms in introns are reported only if variant was seen in more than one unrelated individual.

dGTP and dATP are extended, demonstrating that both the G/C and A/T alleles are expressed in the lung tumor samples. The silent A/T allele in normal lung tissue is clearly activated in lung tumor tissues. These results provide, to our knowledge, the first evidence that activation of a *p73* silent allele is associated with lung carcinogenesis.

The discovery of allelic activation and increased expression of the *p73* gene in lung tumors encouraged us to challenge the hypothesis that p73 might play a role as a tumor suppressor (5–7). We first isolated the intron/exon boundary sequences for all 13 coding exons of *p73* gene by vectorette-PCR (14) and designed primers from the corresponding intron sequences for amplification of each exon. We then screened 21 lung tumor DNA samples for *p73* mutations using the DHPLC method (15, 16) and PCR direct sequencing. However, no *p73* mutations were identified in these tumor samples or in several other types of tumors screened (data not shown). However, we did observe a number of polymorphisms in exons 2, 5, 9, and 14 and in introns 6, 8, and 12 (Table 2). Thus, our data do not support the theory that p73 acts as a p53-like tumor suppressor.

Activation of an imprinted allele in tumor (LOI) has been described for several imprinted genes, such as *IGF2, H19, IPW,* and *p57KIP2* (17–20). For *H19* and *IGF2,* LOI has been observed in several kinds of tumors and has been correlated with tumorigenesis (21, 22). LOI of *IGF2* gene has been observed in 77% of Wilms' tumors (17). Similarly, LOI of the *H19* gene has been described in 50% of esophageal cancers (19) and LOI of the *p57KIP2* gene in 10% of Wilms' tumors (18). p73 had previously been shown to be monoallelically expressed. Here, we have demonstrated activation of the *p73* silent allele in lung tumors. Although the imprinting status for *p73* is still unknown, activation of the *p73* silent allele in tumors seems to have a similar effect as LOI. However, in our studies, activation of a silent allele was observed in all five heterozygous tumor samples (100%). This may indicate that *p73* may play a similar but more important role than LOI in tumorigenesis. The activation of *p73* silent allele, unlike the effect of LOI, which is limited to certain types of tumors, may be a general epigenetic modification involved in the development of many different tumor types. Revealing the epigenetic modification mechanism and the imprinting status of the *p73* gene will help to further understand the real function of p73.

All of our experiments lead us to believe that p73 may not function as a tumor suppressor. However, overexpressed wild-type p73 may function as a p53 dominant-negative protein in lung tumorigenesis. For example, codon 157 (GTC, Val) of p53 is a mutational hot spot specific for lung cancer. This codon in wild-type p73 is ATC (Ile). Therefore, wild-type p73 when overexpressed may mimic mutant p53. Another possibility first proposed by Kaghad *et al.* (4) is that disruption of normal p53 function, as is observed in the majority of lung cancers, results in compensatory or deleterious up-regulation of p73 expression. Thus, either mutant p53 or reduction of p21 may trigger an increase of p73 expression. This may therefore explain why p73 is highly expressed in the tumor tissues analyzed, why the silent allele of *p73* is activated, and also why there were no detectable mutations in the *p73* gene in the tumor samples.

Acknowledgments

We thank Dr. D. James for advice.

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