Elevated and Biallelic Expression of p73 Is Associated with Progression of Human Bladder Cancer

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Abstract

p73, a member of the p53 family at 1p36.3, has been demonstrated to be expressed monoallelically and induce apoptosis or G1 arrest of the cell cycle. To explore the candidacy of p73 as a suppressor in bladder tumorigenesis, we examined expression level, allelic origin, and mutation of p73 mRNA in 45 primary bladder carcinomas. Quantitative PCR analysis showed no allelic loss of the gene but showed various levels of mRNA expression in both carcinoma and noncancerous tissues. Elevated expression of p73 was frequently observed in carcinoma tissues [18 (40.0%) of 45] and showed a strong correlation with tumor stage or grade. Alleotyping analysis using a Sty1 polymorphism detected biallelic expression in 12 (52.2%) of 23 heterozygous carcinomas but none in 4 noncancerous tissues. Tumor-specific biallelic expression was also identified from one matched set. In addition, 8 (66.7%) of these 12 expressed high levels of p73 mRNA, whereas only 2 (18.2%) of 11 monoallelic expressors showed high matched set. In addition, 8 (66.7%) of these 12 expressed high levels of p73 mRNA, whereas only 2 (18.2%) of 11 monoallelic expressors showed high expression, which suggests that the increased expression of p73 might be caused by the transcriptional activation of a silent allele in carcinomas.

Introduction

Recently, a p53-related gene, termed p73 gene, was identified at chromosome 1p36.3, which is frequently deleted in a variety of human cancers including neuroblastoma and melanoma (1). p73 encodes a protein with significant homology to the p53 tumor suppressor through its DNA-binding, transactivation, and oligomerization domains. Al- lelic loss and monoallelic expression in cell lines and tumor specimens supported the notion that p73 could be a tumor suppressor gene at 1p36. Recent in vitro studies showed that overexpression of p73 can induce apoptotic cell death and G1 cell cycle arrest and lead to the transcriptional activation of the p53 cellular target genes including p21(Waf1), which suggests that p73 could have some characteristics similar to p53 (2, 3).

Despite a striking similarity to p53, p73 is not up-regulated in response to DNA damaging agents known to induce p53 and regulates cellular p53 target genes differentially, both of which suggest that the p53 and p73 signaling pathways may be involved in the cellular response to different stimuli (2, 3). Studies showed that none of the p53-inactivating viral oncoproteins, such as adenovirus E1B 55K, SV40 T antigen, and human papillomavirus E6, destabilize p73, and inactivation of p73 is not required for transformation (4, 5).

In addition, to date, no mutations have been identified in the remaining p73 allele in tumors showing loss of heterozygosity at 1p36 (6–9), which raises the possibility that p73 is not a tumor suppressor gene and is not the relevant target of 1p36 deletions.

To explore the potential involvement of p73 alterations in the pathogenesis of bladder cancer, we investigated the expression level, allelic origin, and mutation of p73 mRNA in 45 primary bladder carcinomas compared with 8 noncancerous tissues, with reference to the mutational status of p53 and expression of p21(Waf1) and MDM2. Here, we first report that abnormal elevation of p73 expression due to the activation of a silent allele is associated with the progression of human bladder cancer.

Materials and Methods

Tissue Specimens and Human Cell Lines. Forty-five carcinoma and 8 noncancerous bladder tissue specimens including 4 matched sets were obtained from 45 bladder cancer patients and 4 noncancerous patients by surgical resection in the Kyung Hee University Medical Center (Seoul, Korea). Tissue specimens were snap-frozen in liquid N2 and stored at −70°C until used. Six human cell lines, derived from neuroblastoma (IMR32 and SK-N-SH), breast carcinoma (MCF7), lymphoma (HL60 and U937), and bladder carcinoma (J82) were also included to validate the quantitative RT-PCR2 approach for p73 expression. Extraction of total cellular RNA and cDNA synthesis were performed as described previously (10). Genomic DNA was extracted from the same cells of the tissues from the DNA phase after RNA was extracted.

Quantitative PCR Analysis. For quantitative evaluation by PCR, we initially performed the PCR reaction over a range of cycles (20, 24, 28, 32, 36, and 40 cycles). Diluted (1:4) cDNA (12.5 ng/50 μl PCR reaction) undergoing 28–36 cycles was observed to be within the logarithmic phase of amplification and yielded reproducible results with the primers used for p73; p73-1 (sense: 5′-ATGCCGGGGTGTCGGCGACGC-3′) and p73-14 (antisense: 5′-GCCATGACATGATCTAGTGC-3′) and an endogenous expression standard gene GAPDH (10, 11). For detection of alternatively spliced α and β variants of p73 transcripts, primers p73-1 (sense: 5′-GACCGAAAAGCT-GATGAGGACC-3′) and p73-9 (antisense: 5′-AGATGTTGCTAGG-GTCGTGAC-3′) were used. PCR was performed for 32 cycles at 95°C (1 min), 60°C (0.5 min), and 72°C (1 min) in 1.5 mM MgCl2-containing reaction buffer (PCR buffer II, Perkin-Elmer-Cetus). Ten μl of RT-PCR products were resolved on 2% agarose gels and subjected to Southern blot analysis to confirm the specificity of amplification using a biotinylated, internally positioned oligonucleotide primer p73-12 (antisense: 5′-AGAGACAGCAGAGG-TGCCTGC-3′) as a probe and chemiluminescence detection. Quantitation of expression levels was achieved by densitometric scanning of the ethidium bromide-stained gels. Absolute area integrations of the curves representing each specimen were then compared after adjustment for GAPDH expression. For quantitative DNA-PCR analysis of the p73 gene, 200 ng of genomic DNA was used for amplification of exon 2 of the gene with an intron-specific primer p73-E2S (sense: 5′-CAOGCCACATCGGCTGCC-3′) and p73-12 (antisense: see above). The primers for c-N-Rax used for an endogenous control for quantitative DNA-PCR (RS60 and RS61) were reported previously (12). Integration and analysis were performed using the Molecular Analyst software program (Bio-Rad, Hercules, CA).

Received 1/14/99; accepted 4/28/99.

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2 The abbreviations used are: RT-PCR, reverse transcription-PCR; SSCP, single-strand conformational polymorphism; RFLP, restriction fragment length polymorphism.
Allelic Expression of p73. Syl polymorphism in exon 2 was used to distinguish two distinct alleles (1). The p73 exon 2 region of each tissue specimen was amplified using primers p73-E25 and p73-12 (for DNA-PCR) or p73-1 and p73-14 (for RT-PCR). One µl of the first RT-PCR products was used as template for nest-PCR with primers p73-13 (sense: 5'-GGGCTGCG-GACGGTGTGAGAC-3') and p73-12 (antisense: see above). Twenty µl of the PCR products were then digested with a restriction endonuclease Syl (New England Biolabs, Inc., Beverly, MA) overnight and analyzed on a 3% agarose gel. To confirm the results, 20 µl of the undigested PCR products were also subjected to SSCP analysis as described below.

Nonisotopic RT-PCR-SSCP Analysis. Nonisotopic RT-PCR-SSCP analysis was performed as described previously (10). The entire coding region of p73 transcript was initially amplified with primers p73-1 (sense: see above) and p73-10 (antisense: 5'-CTCCTGGGAGGTGGTTGACAC-3') and then nest-PCRs with six sets of primers were done using 1 µl of the PCR products as templates. The primer pairs used for nest-PCR were:

(a) p73-13 (sense: see above) and p73-14 (antisense: see above);
(b) p73-7 (sense: 5'-GGGCTGCG-GACGGTGTGAGAC-3') and p73-8 (antisense: 5'-ATGCGGAAGAAGTGCTC-3');
(c) p73-7 (sense: see above) and p73-2 (antisense: 5'-GGGCTGCG-GACGGTGTGAGAC-3');
(d) p73-3 (sense: 5'-CTCCTGGGAGGTGGTTGACAC-3') and p73-4 (antisense: 5'-GGGCTGCG-GACGGTGTGAGAC-3');
(e) p73-11 (sense: see above) and p73-9 (antisense: see above); and
(f) p73-5 (sense: 5'-GGGCTGCG-GACGGTGTGAGAC-3') and p73-6 (antisense: 5'-CCAGGGAGCGGGGGCTAATGACT-3').

The PCR products of over 300 bp in lengths were digested with endonuclease(s) to increase the sensitivity of SSCP analysis (10). Twenty µl of the PCR products were then digested with a restriction endonuclease Sty and subjected to SSCP analysis as described below.

Validation of Quantitative RT-PCR. For validation of our RT-PCR approach for quantitative discrimination of p73 expressions, levels of p73 mRNA expression were evaluated in five human cell lines in which the genomic status and mRNA expression of p73 were previously characterized (1). Two lymphoma cell lines, U937 and HL60, which indicated that the p73 mRNA expression was characterized. Consistent with a previous report (1), various levels of p73 expression were observed from three expressor cell lines (p73/c-N-Ras: IMR32, 0.52; SK-N-SH, 1.11; MCF7, 1.53) and the J82 bladder carcinoma cell line showed strong expression of p73 mRNA (1.20).

Elevated Expression of p73 in Bladder Carcinomas. To determine the expression levels of p73 in bladder tissue specimens, cDNAs prepared from 8 noncancerous and 45 carcinoma tissues were subjected to PCR under the same condition used for the cell lines. Highly reproducible results were obtained from three independent quantitative PCRs and the mean of expression levels was obtained. As shown in Fig. 1, various levels of p73 expression (0.10 –1.74) were observed in 45 carcinoma tissues, whereas expression levels in 8 noncancerous tissues were in a range of 0.12 –0.55. This result in noncancerous bladder tissues is in contrast to the recent observation of interindividual differences in p73 expression in lung tissues (6). Thus, we arbitrarily set expression levels over 1.10 (two times the noncancerous maximum) as high expression, and 18 (40.0%) of 45 carcinomas were classified as high expressers. Interestingly, a positive correlation of high expression of p73 with tumor stage or grade was recognized (Table 1). Although 8 (29.6%) of 27 T1-T2 tumors showed high expression, 2 (40.0%) of 5 T2 and 8 (61.5%) of 13 T3-T4 tumors were identified as high expressers. High expression was also observed in 4 (22.2%) of 18, 5 (35.7%) of 14, and 9 (69.2%) of 13 grade I, II, and III tumors, respectively. Expression of an alternatively spliced variant p73b, which lacks exon 13 at the COOH terminus, was extremely low or nearly undetectable in all of the specimens that we examined.

Genomic Status of p73. To investigate whether the expression levels of p73 mRNA are related to the genomic status of the p73 gene, quantitative DNA-PCR analysis was performed with genomic DNA of the same control cell lines and tissues from which p73 mRNA expression was characterized. Consistent with a previous report (1), various levels of the p73 gene were detected in three control cell lines (p73/c-N-Ras: IMR32, 0.52; SK-N-SH, 1.11; MCF7, 1.53) and the J82 bladder cell line (1.18). However, none of the 53 specimens, including 8 noncancerous tissues, showed significantly low or high levels of p73 suggesting genomic alteration of the gene (Fig. 1).

Results and Discussion

Table 1 Expression level and allelic type of p73 mRNA in bladder tissues.

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| Numbers in parentheses are percentage.
| * N: normal; Ca: carcinoma; High exp., high expression.
| + Expression level (p73/GAPDH) of each specimen was determined as the mean of three quantitative RT-PCR results (Normal, 0.12–1.10; High, >1.10).
PCR products covering the exon 2 region of p73, Lanes T1-SH, and MCF7) were included as allotype controls. Biallelic expression was shown in StyG/C specimens showed low or nearly undetectable levels of p53 no correlation with the expression level of p73.

Expression of p73 in Bladder Carcinomas. Monoaallelic expression of p73, probably due to the genomic imprinting of a paternally transmitted allele, has been described in neuroblastoma (1). To investigate the allelic expression pattern of p73 in bladder tissues, we performed RFLP analysis of DNA- and RT-PCR products using a StyI polymorphism in exon 2 of the gene (1, 8). The cell lines IMR32, SK-N-SH, and MCF7, which have been reported to express A/T, A/T, and G/C allele, respectively, were included as allotype controls (1). As shown in Fig. 2, the expected patterns of StyI digestion were observed from IMR32 and MCF7. However, biallelic expression was observed from SK-N-SH, which has been previously reported as a monoallelic expressor. Only G/C transcripts were found from the J82 bladder carcinoma cell line. StyI RFLP analysis of genomic PCR products identified 4 (50.0%) of 8 noncancerous and 23 (51.1%) of 45 carcinoma tissues as heterozygous. Interestingly, although 12 (52.2%) of 23 informative carcinomas were identified as biallelic expressors, none of 4 informative noncancerous tissues showed biallelic expression (<0.05). Furthermore, 8 (66.7%) of 12 biallelic expressors were found to express high p73 mRNA, whereas only 2 (18.2%) of 11 monoallelic expressors showed high expression, which strongly suggests that elevated expressions of p73 mRNA in carcinomas may be caused by the transcriptional activation of a silent allele in these tissues. Our observation of frequent biallelic expression of p73 in carcinoma tissues is consistent with a recent study demonstrating that the loss of imprinting or the switching of allelic expression of the p73 gene is associated with the development of renal cell carcinomas (13).

No Mutation of p73 Transcripts. To analyze the mutational alterations of p73 transcripts, we performed RT-PCR-SSCP analysis of the entire coding region of the transcript. SSCP analysis with PCR products amplified with six sets of primers, digestion of the same PCR products with different restriction endonuclease(s), and electrophoresis under two different running conditions failed to detect any types of mutation leading to amino acid substitutions or frameshifts, except for previously described polymorphisms (6, 8). This result indicates that, unlike p53, mutational alteration of p73 may not be a main genetic event for the bladder carcinogenesis.

No Correlation of p73 Expression with p53 Status or p21Waf1 Expression. To evaluate the possible relationship of the expression status of p73 with p53 mutation in carcinomas, we investigated the expression and sequence alteration of p53 and expression of p21Waf1 and MDM2 using a quantitative RT-PCR, RT-PCR-SSCP, and direct sequencing analysis (10, 14). Loss of mRNA expression or sequence alteration of p53 was identified in 12 (26.7%) of 45 carcinomas, but no correlation with the expression level of p73 was observed (data not shown). In addition, although 9 (75.0%) of 12 mutant p53-carrying specimens showed low or nearly undetectable levels of p21Waf1 expression, 7 (25.9%) of 27 normal and 4 (22.2%) of 18 high p73 expressers were found to express low p21Waf1 mRNA, which indicates that there is no correlation of p73 expression with the basal transcription of the p21Waf1 gene. Also, no association was recognized between expressions of p73 and MDM2.

Our observation of increased and biallelic expression of p73 in bladder tumors compared with noncancerous tissues, its correlation with tumor stage or grade, and no allelic alteration or mutation of the gene strongly suggests that p73 is unlikely to be a tumor suppressor gene that conforms to a two-hit model of tumorigenesis. Also, these data are well consistent with recent findings of a more intense expression of p73 in lung, prostate, and neuroblastoma tumors than in normal tissues, which suggests that the activation of a silent allele or overexpression of p73 rather than p73 as tumor suppressor may contribute to the tumorigenesis (6–9). Recently, Marin et al. (4) also demonstrated that p73 is not destabilized by viral oncoproteins, and high levels of p73 are expressed in cells transformed by SV40 T antigen or adenovirus E1B, which raises the possibility that the preservation of p73 functions renders a cell permissive for viral replication or transformation (4, 5). Taken together, these considerations suggest that, despite its high degree of structural similarity to p53 and a role by epigenetic control of expression, there is presently no genetic evidence that the inactivation of p73 is required for the transformation or malignant progression of human tumors.

In summary, this study strongly indicates that p73 is not a target of genetic alteration in bladder tumorigenesis. Elevated and frequent biallelic expression of wild-type p73 in carcinomas compared with noncancerous tissues suggests that p73 may contribute to the progression of bladder tumors through its transcriptional activation. Additional studies will be required to assess the biological significance of altered p73 expression in the growth of bladder tumors.

References

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