Mutational and Expression Analysis of the p73 Gene in Melanoma Cell Lines

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ABSTRACT

A novel p53-related gene, p73, was recently isolated and cytotogenetically mapped to chromosome region 1p36. Functionally, p73 expression induces p21wild and suppresses tumor cell growth. We mapped p73 using radiation hybrids and localized the gene to an interval that putatively harbors a melanoma tumor suppressor locus. We then analyzed p73 transcripts from 24 melanoma cell lines using reverse transcription-PCR/single strand conformation polymorphism and identified nine polymorphic sequence changes (three novel and six previously published polymorphisms); furthermore, we found evidence of biallelic transcription in our cell lines. However, we did not detect any deleterious mutations. These data suggest that the p73 gene is unlikely to be essential in melanoma tumorigenesis.

INTRODUCTION

Several lines of evidence support the existence of a tumor suppressor gene involved in melanoma tumorigenesis on chromosome region 1p36. Cytogenetic studies of sporadic melanoma specimens have documented frequent structural abnormalities and imbalances in this region (reviewed in Ref. 1, Ref. 2). Further evidence for a melanoma tumor suppressor in this region comes from loss of heterozygosity studies reporting allelic loss at 1p36 in up to 43% of melanomas (3). Finally, a subset of melanoma-prone kindreds, which lack germline CDKN2A mutations, demonstrate linkage to 1p36 markers, suggesting the presence of an unidentified heritable tumor suppressor at this location (4–7). Abnormalities in chromosome region 1p36 have been described in many cancers, especially neuroblastoma. The most frequently observed genetic alterations in neuroblastomas are deletions of 1p (8, 9). The best evidence suggests that the neuroblastoma and melanoma neuroblastoma regions of loss are concordant. Likewise, changes within this region have also been reported in ovarian, breast, and colon cancer (10–12).

Recently, Kaghad et al. (13) isolated a p53-related gene, p73, which was cytotogenetically mapped to 1p36. On the basis of its sequence homology to p53 and its ability to induce p21wild and suppress cell growth, p73 is positioned functionally and functionally an attractive tumor suppressor candidate. Although initial analysis of neuroblastoma cell lines failed to reveal p73 mutations, Kaghad et al. (13) suggested that monoallelic transcription of p73 and absence of protein expression in these neuroblastoma cell lines were consistent with involvement in tumor development (13). Of note, several studies have documented a low rate of p53 alterations in melanoma (14–17), thereby raising the possibility that the mmum target for mutations in melanoma is another p53-related gene. Taken together, p73 is both an attractive positional and functional candidate for the 1p36 tumor suppressor gene implicated in melanoma pathogenesis.

To test the potential involvement of p73 in melanoma, we confirmed and more precisely mapped its location using radiation hybrids

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and subsequently used RT-PCR-SSCP4 analysis to examine 24 melanoma cell lines for mutations in the p73 transcript.

MATERIALS AND METHODS

Cell Lines. The 24 human melanoma cell lines have been described previously (18). Of these 24 melanoma cell lines, 21 were evaluable for CDKN2A/CDK4, and 19 showed evidence of CDKN2A inactivation (mutation or homozygous deletion) or CDK4 activation (mutation at codon 24 or codon 22) (18, 19). For expression analysis by FUDR digestion, 12 peripheral lymphocyte cell lines from melanoma families were provided by Dr. Alisa Goldstein (National Cancer Institute, Bethesda, MD) and 9 colon cancer cell lines obtained from American Type Culture Collection (Rockville, MD): COLO 320, HT-29, LS 174T, SK-Co-1, SW48, SW403, SW480, SW620, and SW948. Cells were grown in DME supplemented with 10% FCS and antibiotics.

RH Mapping. Stanford G3 RH panels were purchased from Research Genetics, Inc. (Huntsville, AL). DNAs from all 83 RH clones were screened for human p73 by PCR using Advantage-GC Genomic PCR kit (Clontech, Palo Alto, CA) and primers p73-8F and p73-8R (see below). PCR was performed as recommended in the manufacturer’s manual by 30 cycles of 94°C for 30 s and 68°C for 180 s. The raw data obtained were submitted to Stanford Human Genome Center for linkage analysis.

RT-PCR-SSCP and Sequence Analyses. Total RNA was isolated from human melanoma cell lines using TRIZOL reagent (Life Technologies, Inc., Gaithersburg, MD). First-strand cDNAs were synthesized from 2 μg of total RNA using SUPERSCRIPT II RNase H− reverse transcriptase and oligo(dT)23−33 primer (Life Technologies) as follows the protocol recommended for GC-rich templates. For RT-PCR-SSCP screening, p73 cDNA was amplified into nine overlaying fragments ranging from 235 to 344 bp using Advantage-GC cDNA PCR kit (Clontech). Primer pairs were: P73-1F, 5′-AGGGGACGCAAGCAACGGGG-3′ and P73-1R, 5′-GCTGCTCTCATCTCAGTGGCTG-3′; P73-2F, 5′-GGGCTGACATCATCTCTGGGAC-3′ and P73-2R, 5′-GGGTGAGCACCTTTGATCTG-3′; P73-3F, 5′-CAAGCCTTGTGGGTCTGACCTGAC-3′ and P73-3R, 5′-CAGGAGGCTGCAACAGCAGCAG-3′; P73-4F, 5′-GCTGAGGACCTGGTCTAGTGGTACAGTGTGATC-3′ and P73-4R, 5′-GCTGAGGACCTGGTCTAGTGGTACAGTGTGATC-3′; P73-5F, 5′-GCTGAGGACCTGGTCTAGTGGTACAGTGTGATC-3′ and P73-5R, 5′-GCTGAGGACCTGGTCTAGTGGTACAGTGTGATC-3′; P73-6F, 5′-GCTGAGGACCTGGTCTAGTGGTACAGTGTGATC-3′ and P73-6R, 5′-GCTGAGGACCTGGTCTAGTGGTACAGTGTGATC-3′; P73-7F, 5′-GCTGAGGACCTGGTCTAGTGGTACAGTGTGATC-3′ and P73-7R, 5′-GCTGAGGACCTGGTCTAGTGGTACAGTGTGATC-3′; P73-8F, 5′-GCTGAGGACCTGGTCTAGTGGTACAGTGTGATC-3′ and P73-8R, 5′-GCTGAGGACCTGGTCTAGTGGTACAGTGTGATC-3′; P73-9F, 5′-GCTGAGGACCTGGTCTAGTGGTACAGTGTGATC-3′ and P73-9R, 5′-GCTGAGGACCTGGTCTAGTGGTACAGTGTGATC-3′.

The raw data obtained were submitted to Stanford Human Genome Center for linkage analysis.

TA Cloning and Sequence Analyses. The p73 cDNA containing several sequence polymorphisms was first amplified from RT products using AmpliCycle sequencing kit (Perkin-Elmer, Foster City, CA).

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1 The abbreviations used are: RT-PCR-SSCP, reverse transcription-PCR/single-strand conformation polymorphism; RH, radiation hybrid.

2 H. Tsa0 and F. G. Haluska, unpublished data.

4 The abbreviations used are: RT-PCR-SSCP, reverse transcription-PCR/single-strand conformation polymorphism; RH, radiation hybrid.
Sequence Polymorphisms and Lack of Mutations in Melanoma Cell Lines. Using RT-PCR-SSCP, we analyzed p73 transcripts from 24 melanoma cell lines and detected 9 polymorphic single-base substitutions. Fig. 1 shows the mobility shifts for a novel polymorphism from three of our lines (Lanes 2, 7, and 10). Sequence analysis revealed a silent C-to-T polymorphism at the third position of codon 173 (Fig. 2). Two other novel polymorphisms in the 3′ untranslated region were also found (2136 G/A and 2172 A/C). In addition to the double nucleotide substitution at nucleotides 4 (A/G) and 14 (T/C) of exon 2 (13), silent polymorphisms at codons 336 (C/T), 349 (T/C), 557 (G/A), and 610 (G/A) were detected and have been reported previously in lung cancer specimens (23). A compilation of sequence alterations found in this study are denoted in Fig. 3. Examination of melanoma and colon cancer cell lines by Southern blotting revealed no evidence of rearrangements when the full-length p73 cDNA was used as probe (data not shown).

Of note, five single-base substitutions: C to T at codon 336, T to C at codon 349, G to A at codon 557, G to A at codon 610, and A to C at nucleotide 2172 in 5′ untranslated region were concurrently present in two melanoma cell lines, MGH-G-MEL and MGH-PO-1, and one peripheral blood lymphoblastoid line. Cloning of each transcript followed by sequence analysis of p73 cDNA clones indicated that all of the five substitutions were on the same allele. This cluster of multiple base substitutions on one allele suggests that they were derived from a common ancestral chromosomal region.

Biallelic Expression of p73 in Melanoma Cell Lines and Other Tissues. In certain melanoma cell lines, transcripts for both alleles of p73 can be identified (Fig. 1, Lane 2), thereby suggesting a lack of allelic expression imbalance. Overall, 6 of 24 melanoma cell lines showed evidence of biallelic transcription in RT-PCR-SSCP analysis (data not shown). The remaining cell lines may be homozygous for the p73 locus or hemizygous due to chromosomal loss. Thus, the frequency of heterozygous p73 transcripts may be an underestimate of the true rate of biallelic transcription in our lines.

The G-to-A transition at codon 557 eliminates an HhaI site, allowing discrimination between polymorphic transcripts using restriction digestion of RT-PCR products (Fig. 4A). In addition to the two melanoma cell lines (MGH-G-MEL and MGH-PO-1), one colon cancer cell line (LS 174T) and two peripheral lymphoblastoid cell lines from members of chromosome 1p36-linked melanoma-prone kindreds (3838, 6169) showed biallelic transcription (Fig. 4B) using this Hha-I sensitive detection method. Of the 24 melanoma, 9 colon cell lines, and 12 peripheral lymphocytic cell lines analyzed by genomic PCR-SSCP, only 5 were informative for the polymorphic HhaI site (data not shown), and all of these showed evidence of biallelic expression (Fig. 4B).

Deleterious mutations in p73 have yet to be identified in neuroblastosmas (13), prostate cancers (24), or lung cancers (23). With this study, we establish that p73 mutations are also rare, if existent, in melanomas. One explanation for the failure to detect mutations is that of technical inadequacies using the RT-PCR-SSCP approach. This is unlikely the reason because we were able to detect numerous conformation-sensitive sequence alterations with our polymorphisms. Alternatively, the polymorphic changes may lead to secondary mRNA
structures unfavorable for protein synthesis. In neuroblastoma cell lines, for instance, an absence of p73 protein is found despite wild-type transcript sequences (13). The relationship between secondary transcript structure and translation remains to be established. Finally, type transcript sequences (13). The relationship between secondary transcript structure and translation remains to be established. Finally, type transcript sequences (13). Versteeg et al. (25) demonstrated that p73 can induce the expression of a novel p53 isoform in response to genotoxic stress. The biological significance of this finding is currently being elucidated.

Although p73 is an attractive tumor suppressor candidate by both genetic and functional criteria, it remains to be determined whether it plays a critical role in the development of melanoma along with other cancers. Further research is needed to elucidate the role of p73 in the pathogenesis of melanoma and other cancers.

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