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Mutational and Expression Analysis of the *p73* Gene in Melanoma Cell Lines[1]

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ABSTRACT

A novel p53-related gene, *p73*, was recently isolated and cytogenetically mapped to chromosome region 1p36. Functionally, *p73* expression induces $p21^{waf}$ 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 germ-line *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 melanoma and 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 cytogenetically mapped to 1p36. On the basis of its sequence homology to *p53* and its ability to induce $p21^{waf}$ and suppress cell growth, *p73* is positionally 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 neuroblastomas 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 true 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 and subsequently used RT-PCR-SSCP[4] 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).[5] For expression analysis by *Hha*I 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 DMEM 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$(dT)_{12-18}$ (Life Technologies) by following the protocol recommended for GC-rich templates. For PCR-SSCP screening, p73 cDNA was amplified into nine overlapping fragments ranging from 235 to 344 bp using Advantage-GC cDNA PCR kit (Clontech). Primer pairs were: P73-1F, 5′-AGGGGACGCAGCGAAACCGGGG-3′ and P73-1R, 5′-GCTGCTCATCTGGTCCATGGTGCTG-3′; P73-2F, 5′-GGGCATGACTACATCTGTCATGGCC-3′ and P73-2R, 5′-GGGTGGACACCTTGATCTGGATGGG-3′; P73-3F, 5′-CCAAGTCAGCCACCTGGACGTACTC-3′ and P73-3R,5′-CACCTGTGGTGGCTCATAGGGCACC-3′;P73-4F,5′-CTCTCGCAGTATGTGGATGACCCTG-3′ and P73-4R, 5′-TCTGCTTGAAGGCACGCTTGCTGGC-3′; P73-5F, 5′-GATGAGGACCACTACCGGGAGCAGC-3′ and P73-5R, 5′-GTGTAGGTGACTCGGCCTCTGTAGG-3′; P73-6F, 5′-AGCCTGGAGCTGATGGAGTTGGTGC-3′ and P73-6R, 5′-GAGTGCAGTGGGACCCCGAGACCAT-3′; P73-7F, 5′-AACGGCGAGATGAGCAGCAGCCACAG-3′ and P73-7R, 5′-AGTTCCCCTGAGCCGCCGATGGAGAT-3′; P73-8F, 5′-CTGAAGCAGGGCCACGACTACAGCAC-3′ and P73-8R, 5′-CCGTGAACTCCTCCTTGATGGGCTGC-3′; P73-9F, 5′-TGACGAGTGGGCGGACTTCGGCTTC-3′ and P73-9R, 5′-TGCAGGTGACTCAGGCTGTCCACAGG-3′.

Amplification was carried out in 10-μl reaction mixtures containing 1 μl of RT product and 2 μCi of [α-^{32}P]dCTP (NEN, Boston, MA) through 40 cycles of 94°C for 30 s and 68°C for 180 s. Denatured DNA fragments were electrophoresed through two 0.5× MDE gels (FMC BioProducts, Rockland, ME) in 0.6× TBE, one with 5% glycerol and the other without glycerol, for 16 h at room temperature at 5 W (for glycerol plus gel) or 4 W (for glycerol minus gel). DNA fragments showing mobility shifts were then prepared by PCR under the same condition, separated on agarose gel, purified using QIAquick kit (Qiagen, Inc., Santa Clarita, CA), and directly sequenced using AmpliCycle sequencing kit (Perkin-Elmer, Foster City, CA).

TA Cloning and Sequence Analysis. The p73 cDNA containing several sequence polymorphisms was first amplified from RT products using Advantage-GC cDNA PCR kit (Clontech) with primers P73-1F and P73-9R and subsequently cloned into pCR2.1 TA-vector (Invitrogen, Carsbad, CA) and directly sequenced as described above.

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

[5] H. Tsao and F. G. Haluska, unpublished data.

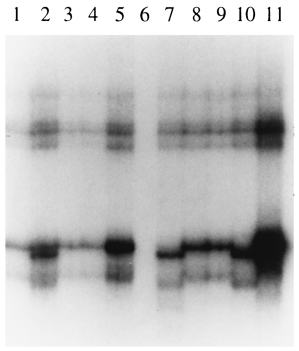

Fig. 1. RT-PCR-SSCP analysis of melanoma cell lines. Total RNAs from melanoma cell lines were reverse transcribed, amplified using primers P73-3F and P73-3R, and subjected to SSCP analysis. *Lanes 1–5* and *7–10* represent various melanoma cell lines, whereas *Lane 11* is a normal control. *Lanes 7* and *8* show contrasting homozygous patterns suggestive of two alternative sequences. *Lane 2* shows both patterns, suggestive of biallelic transcription.

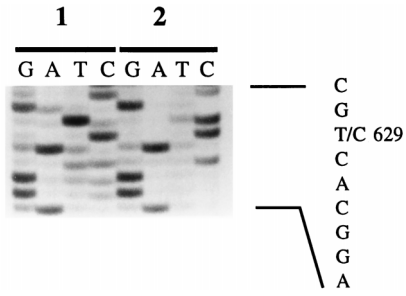

Fig. 2. Sequence analysis. PCR sequencing of fragments exhibiting mobility shifts shown in Fig. 1 (*Lanes 7* and *8*) reveals a nucleotide 629 T/C polymorphism at the third position of codon of 173 (Thr).

Analysis of Allele Expression. RT-PCR products specific for p73α transcript were prepared by 40 cycles of amplification using Advantage-GC cDNA PCR kit (Clontech) and primers P73U3A (5′-GCAGAACCTGACCATTGAG-GACCTGG-3′) and P73D3A (same as P73-7R), purified using QIAquick kit (Qiagen), digested with restriction endonuclease *Hha*I (New England Biolabs, Inc., Beverly, MA), and analyzed on 2% agarose gels.

RESULTS AND DISCUSSION

RH Mapping of p73. p73 was originally mapped to chromosome 1p36 using the fluorescence *in situ* hybridization technique (13). Analysis of our RH typing pattern showed linkage to *D1S2893* with a LOD score of 9.28 and physical distance of 19.39 cR 10000. *D1S2893* is in proximity to marker *D1S47* (20), a marker at which loss of heterozygosity has been demonstrated in sporadic melanoma and linkage has been found in a subset of familial melanoma (3, 4). Moreover, Walker (21) found allelic loss on distal 1p between *D1S160* and *D1S243*; *D1S2893*, and thus *p73*, maps to a region delimited by these markers. Finally, *p73* also lies in the commonly deleted region defined in neuroblastomas (22). Thus, our mapping information provides more precise *p73* localization and further confirms the positional candidacy of *p73* in melanoma tumor formation.

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 *Hha*I site, allowing discrimination between polymorphic transcripts using restriction digestion of RT-PCR products (Fig. 4*A*). 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. 4*B*) 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 *Hha*I site (data not shown), and all of these showed evidence of biallelic expression (Fig. 4*B*).

Deleterious mutations in *p73* have yet to be identified in neuroblastomas (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

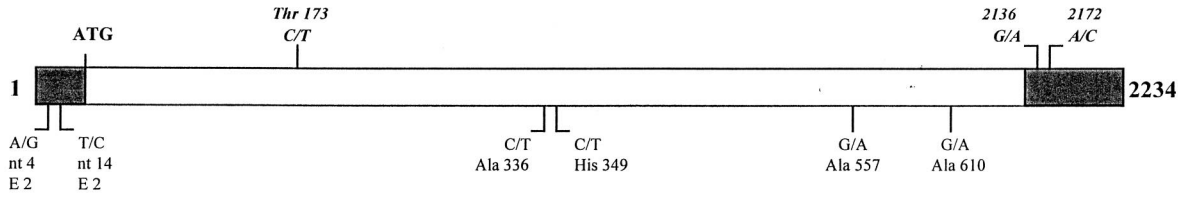

Fig. 3. Summary of *p73* alterations. Nucleotide 1 defines the beginning of the p73 cDNA. *Shaded areas*, 5′ and 3′ untranslated regions. Italicized alterations above the transcript designation are novel polymorphisms identified in this study. Sequence changes below the transcript map were also detected but have been described previously.

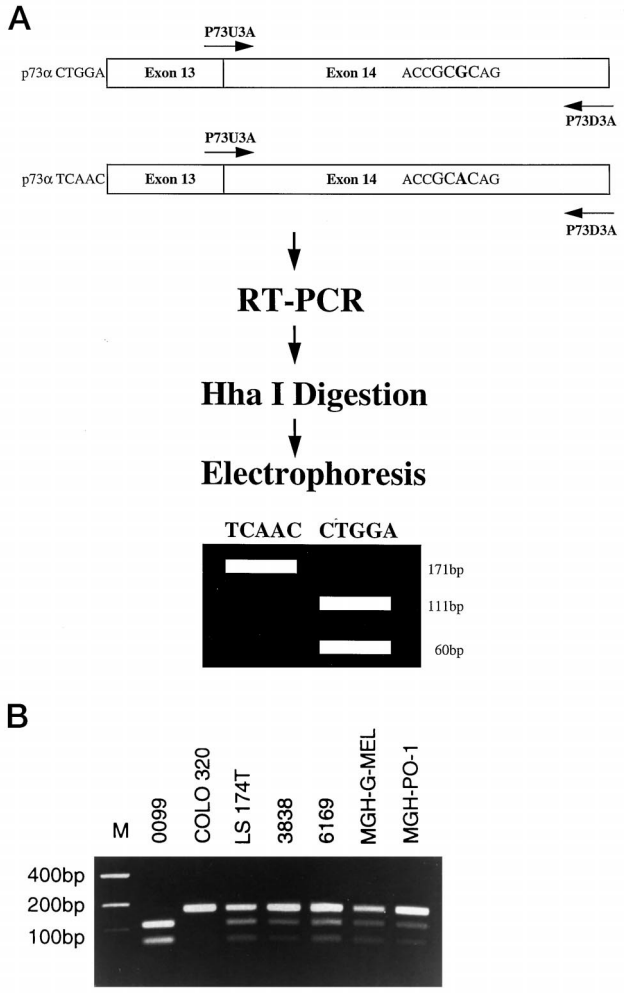

Fig. 4. Expression analysis using an *Hha*I-sensitive polymorphism. *A*, diagram showing detection protocol using sequential RT-PCR and *Hha*I digestion. *B*, demonstration of biallelic expression in different tissues using *Hha*I digestion. Samples 0099 and COLO320 only express one allele, whereas samples LS174T, 3838, 6169, MGH-G-MEL, and MGH-PO-1 express both alleles. MGH-G-MEL and MGH-PO-1 are melanoma cell lines; COLO320 and LS174T are colon cancer lines; 0099, 3838, and 6169 are peripheral lymphoblastoid lines from members of 1p36-linked families.

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, marked allelic expression imbalance through imprinting has been cited as a mechanism of inactivation of *p73* (13). Versteeg *et al.* (25) first reported that in *MYCN* single copy neuroblastomas, most deletions of 1p36 appear to be maternally derived. However, we, along with others (23, 26), have detected biallelic transcription of *p73* in both normal tissue and tumors. These findings represent a departure from results reported for neuroblastoma and suggest that imprinting and monoallelic silencing of *p73* do not play a critical role in the development of melanoma along with other cancers.

Although *p73* is an attractive tumor suppressor candidate by both position and function, lack of definitive mutations in tumors thus far analyzed raises the possibility that inactivation of other genes in this region contribute to the formation of cutaneous melanoma.

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