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Identification and Mutation Analysis of *DOC-1R*, a DOC-1 Growth Suppressor-Related Gene

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The tumor suppressor gene *MEN1* and several oncogenes including *CCND1/cyclin D1/PRAD1* map to chromosome 11q13. However, molecular and cytogenetic analysis suggests the presence of a second tumor suppressor locus at this chromosome region. We have identified a novel gene from chromosome 11q13, which encodes a protein of 126 amino acids sharing an overall 57% identity with the p12^{DOC-1} protein encoded by the *DOC-1* gene, the human homolog of hamster putative tumor suppressor *doc-1* (*d*eleted in *o*ral *c*ancer-*1*). We therefore designated the novel gene as *DOC-1R* for *DOC-1*-related. The cytogenetic location was confirmed by chromosome fluorescent *in situ* hybridization. Northern blot analysis indicated that it was expressed in all the tissues examined. DOC-1R protein showed heterogeneous subcellular localization. RT-PCR-SSCP analysis failed to detect deleterious mutations of the DOC-1R transcript in four premalignant oral keratinocyte lines and 20 different cancer cell lines from tumor types which frequently harbor LOH at chromosome 11q13.

Key Words: **tumor suppressor gene; mutation; DOC-1; DOC-1R.**

Chromosome 11q13 is one of the most common regions involved in genetic alterations observed in human cancers, indicating the pathogenetic importance of the genes at this chromosomal site in the development of human cancers. The multiple endocrine neoplasia type 1 (*MEN1*) locus was mapped to chromosome 11q13 and loss of heterozygosity (LOH) at the locus was found in MEN1-associated tumors, suggesting that *MEN1* might be a tumor suppressor gene (1). Subsequently, the *MEN1* gene was identified by positional cloning and inactivating mutations were detected in familial MEN1 tumors and some sporadic endocrine tumors (2). Several cellular oncogenes, including *CCND1/cyclin D1/PRAD1*, *FGF3/INT2*, *FGF4/HST1*, and *EMS1*, have also been assigned to chromosome 11q13 (3). Alterations of the *CCND1/cyclin D1/PRAD1* gene have been pathogenetically implicated in deregulation of cell cycle associated with several cancer types. Overexpression or amplification of cyclin D1, often correlating with cytogenetically observed homogeneously staining region or double minute chromosomes, has been shown in breast, esophageal, head and neck, and hepatocellular cancers (4–8). However, LOH and cytogenetic studies also suggest the presence of second tumor suppressor locus at chromosome 11q13, and cyclin D1, acting as a dominant oncogene, is not the target of LOH. Using tissue microdissection, Zhuang *et al.* found that 67% (24/36) of *in situ* and 71% (15/21) of invasive breast cancers demonstrated LOH at chromosome 11q13 (9). Jesudasan *et al.* reported deletion and translocation of chromosome 11q13 DNA in 4 of 8 cervical cancer cell lines analyzed (10). More recently, deletion of chromosome 11q13-qter associated with amplification of band 11q13 was detected in head and neck squamous cell carcinomas (HNSCC) (11) and Venugopalan et al defined a tumor suppressor locus implicated in HNSCC to the FGF3/INT2-D11S533 interval (12).

Here we report the identification of a novel gene, DOC-1R, from chromosome 11q13 that is homologous to the DOC-1 gene. *Doc-1, d*eleted in *o*ral *c*ancer-*1,* was originally isolated by subtractive hybridization in a

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hamster oral cancer model (13). Transfection of doc-1 cDNA can suppress some transformed phenotypes of malignant keratinocytes, suggesting that the gene may function as a tumor suppressor. Human DOC-1 gene was mapped to chromosome 12q24 and no mutations have been found in oral and esophageal cancers (14, 15). The $p12^{DOC-1}$ protein has been shown to associate with DNA polymerase α/primase complex and CDK2 thereby raising the possibility that it may be involved in both DNA replication and cell cycle regulation (14). We have identified a DOC-1-related gene from chromosome 11q13 and have performed mutation analysis in various cancer cell lines.

MATERIALS AND METHODS

Cell lines. Three normal human oral keratinocyte cell lines, 4 premalignant oral keratinocyte cell lines and 8 oral squamous cell carcinoma lines have been previously described (16). Five breast cancer cell lines: BT-549, MCF7, MDA-MB-134-VI, MDA-MB-231 and MDA-MB-435S; 4 cervical cancer cell lines: HeLa, Hs 588.T, Hs 636.T and SW756; and 3 ovarian cancer cell lines: Caov-3, SK-OV-3 and SW626 were obtained from American Type Culture Collection (ATCC, Rockville, MD). ATCC lines were grown in Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum and antibiotics.

EST cDNA clones. cDNA clones, from which ESTs AA227129/AA227130, AA401965/AA402084, H62132, N33615/N49458 and R50028/R50367 were generated, were purchased from Research Genetics (Huntsville, AL).

Fluorescent in situ hybridization (FISH). The Keio BAC library was screened by PCR using primers for Whitehead EST marker EST22043 (17). BAC clones obtained after PCR screening were further confirmed by Southern blotting using DOC-1R cDNA as probe. FISH was performed as described (18).

Northern blot analysis. A 12-lane multiple adult human tissue northern blot (Human 12-Lane MTN Blot , Clontech, Palo Alto, CA) was probed using DOC-1R insert from EST cDNA clone for R50028/R50367 as described previously (19). DOC-1R cDNA was labeled by random priming. The same blot was also re-probed with β-actin gene to confirm RNA loading.

GFP construct and transfection. DOC-1R open reading frame was amplified from EST cDNA clone for R50028/R50367 using primers DOC-1R/*Hin*dIII, 5′-CTTCAAGCTTTCCTACAAACCCATCGCCCCTG-3′ and DOC-1R/*Bam*HI, 5′-CGCGGATCCTTAGTGCAACTCGGGATGAAGG-3′. PCR product was digested with *Bam*HI and *Hin*dIII, and then clone into the pEGFP-C3 Vector (Clontech, Palo Alto, CA) at the *Bam*HI and *Hin*dIII sites to generate pEGFP-DOC-1R. The reading frame and sequence of the resulting pEGFP-DOC-1R plasmid was confirmed by cycle sequencing. pEGFP-DOC-1R was transfected into U2OS cells using LIPOFECTAMINE PLUS Reagent (Life Technologies, Gaithersburg, MD). After overnight incubation, fluorescing cells were viewed and photographed using Olympus IX70 Inverted System Microscope (Olympus Optical Co., Ltd., Tokyo, Japan) equipped with FITC filter.

RT-PCR-SSCP analysis. Total RNA was isolated from cultured cancer cells using TRIZOL reagent (Life Technologies, Gaithersburg, MD) and first-strand cDNA was synthesized from 2 μg of total RNA using SUPERSCRIPT II RNase H^- reverse transcriptase and oligo $(dT)_{12-18}$ (Life Technologies, Gaithersburg, MD) by following the protocols recommended. PCR-SSCP was essentially carried out as previously described (20). DOC-1R cDNA was first amplified into 2 overlapping fragments of 324 and 331 bp to cover the entire coding region. The PCR primers are: F1, 5′-CTTGCCAATCAGAGCGCGGCTGAG-3′ and R1, 5′-CTGACAGCAGGTCCGTGTAGGTGC-3′; and F2, 5′-CTACGTGCAGGCGATGAAGCCACC-3′ and R2, 5′-CCAAGGGACACAGGACAGGAAGCC-3′, respectively.

Cycle sequencing. For sequencing of EST cDNA clones, plasmid DNA was purified using QIAGEN's plasmid mini kit (QIAGEN, Inc., Santa Clarita, CA). DNA fragments showing mobility shifts in PCR-SSCP analysis were amplified using Advantage-GC cDNA PCR kit (Clontech, Palo Alto, CA) and gel-purified using QIAquick kit (QIAGEN, Inc., Santa Clarita, CA). Purified plasmids and PCR fragments were sequenced using AmpliCycle sequencing kit (Perkin–Elmer, Foster City, CA).

RESULTS AND DISCUSSION

We searched the NCBI databases for homologs of *DOC-1*. We found two human UniGene clusters with one encoding DOC-1 (Hs. 3436) and another encoding a highly similar protein (Hs. 25664). To obtain the complete coding information, we determined the nucleotide sequences of 5 cDNA clones which contain the ESTs for Hs. 25664 UniGene cluster by cycle sequencing and subjected the assembled sequence of 931 bp (GenBank, AF089814) to the NCBI ORF Finder program. An open reading frame that encodes a protein of 126 amino acids was revealed (Fig. 1A). A polyadenylation signal AATAAA element at nucleotides 903-908 in 3′ untranslated region and an inframe TGA stop codon in 5′ untranslated region suggested that we had obtained a complete cDNA sequence. To find homologues, we searched the NCBI non-redundant protein database with the predicted amino acids sequences using the basic BLASTP program. An overall 57% identity with $p12^{DOC-1}$ was detected (Fig. 1A). We therefore designated this novel gene as *DOC-1R* for *DOC-1-*related gene. Interestingly, the C-terminus shares an 86% identity with $p12^{DOC-1}$ and 48% identity with *C. elegans* protein Y43F4B.7 (Fig. 1B).

The ESTs representing the *DOC-1R* gene have been independently mapped to chromosome 11q13 at interval D11S913–D11S916 by Whitehead and Stanford Human Genome Centers using GB4 and G3 radiation hybrid panels, respectively ((21), http://www.ncbi.nlm.nih.gov/genemap/). The tumor suppressor gene *MEN1* and oncogenes *CCND1/cyclin D1/PRAD1*, *FGF3/INT2*, *FGF4/HST1* and *EMS1* are in this genetic interval. We obtained 5 BAC clones by PCR screening of the Keio library (17) and performed FISH mapping using labeled BAC DNAs as probe (18). All 5 clones exhibited signals at chromosome 11q13. Figure 2 shows a typical twin-spot FISH signals detected using BAC clone KB1574F2. This result further confirmed the map location of the *DOC-1R* locus in the cytogenetic region rich in cancer-related genes. However, one of the five BAC clones, KB1934A8, also revealed strong signals at chromosome 2q11.2 (data not shown) thereby raising the possibility of related sequences on chromosome 2q11.2.

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A

DOC-1R:   1 MSYKPIAPAPSSTPGSSTPGPGTPVPTGSVPSPSGSVPGAGAPFRPLFNDFGPPSMGYVQ 60
            :::::   :    .                ::: ::: :.    . .: :  .:.::::.:: :
DOC-1:    1 MSYKPNLAAHMPAAALNA--------AGSVHSPSTSM-ATSSQYRQLLSDYGPPSLGYTQ 51

DOC-1R:  61 AMKPPGAQGSQSTYTDLLSVIEEMGKEIRPTYAGSKSAMERLKRGIIHARALVRECLAET 120
              .:   :: :  .::..:::.:::::::::::::::::::::::::::::: :::::::::
DOC-1:   52 GTG--NSQVPQSKYAELLAIIEELGKEIRPTYAGSKSAMERLKRGIIHARGLVRECLAET 109

DOC-1R:121 ERNART 126
           ::::::.
DOC-1: 110 ERNARS 115

B

DOC-1R:     74 YTDLLSVIEEMGKEIRPTYAGSKSAMERLKRGIIHARALVRECLAETERNART 126
               : .::..:::.:::::::::::::::::::::::::::::: ::::::::::::::.
DOC-1:      63 YAELLAIIEELGKEIRPTYAGSKSAMERLKRGIIHARGLVRECLAETERNARS 115
               :  :: .:::.::..::::  .:   ::::: :  :. :.: :  : : .  .
Y43F4B.7: 525 YEVLLKLIEEIGKDVRPTYTFNKLTCERLKRNIQAAKVLIRACQQEAETDKKK 576
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FIG. 1. Primary sequence of DOC-1R. (A) The predicted amino acid sequence of DOC-1R protein and its alignment with the DOC-1 encoded $p12^{DOC-1}$. (B) Sequence alignment of DOC-1R C-terminus with $p12^{DOC-1}$ and *C. elegans* protein Y43F4B.7. Numbers indicate amino acid positions. Identical and similar amino acids are highlighted by ":" and "." respectively.

Northern blot analysis using full length cDNA as probe indicated that the *DOC-1R* gene was ubiquitously expressed (Fig. 3). All tissues examined showed two transcripts of about 1.0 and 1.5 kb. Apparently more abundant expression is demonstrated in liver, heart and kidney. The DOC-1 transcript has been found to exhibit similar ubiquitous expression patterns (14, 15).

Immunohistochemical staining using a polyclonal anti-$p12^{DOC-1}$ antibody has demonstrated the nuclear and perinuclear subcellular localization of the $p12^{DOC-1}$ protein (14). We analyzed the amino acid sequences of both $p12^{DOC-1}$ and DOC-1R proteins using the PSORT (Prediction of Protein Sorting Signals and Localization Sites in Amino Acid Sequences) II program (http://cookie.imcb.osaka-u.ac.jp/nakai/psort.html) that is recommended for animal/yeast sequences. The results suggest a nuclear location for both proteins. Furthermore, we engineered a DOC-1R protein fused to the C-terminus of the enhanced green fluorescent protein (pEGFP-DOC-1R) and transfected this construct into human U2OS osteosarcoma cells. As shown in Fig. 4B, pEGFP-DOC-1R expression resulted a heterogeneous distribution compared to the parental pEGFP-C3 vector control (Fig. 4A). Three patterns were visualized: (1). a cytoplasmic and perinuclear pattern (Fig. 4B); (2). a perinuclear (Fig. 4C) pattern; (3). a nuclear and

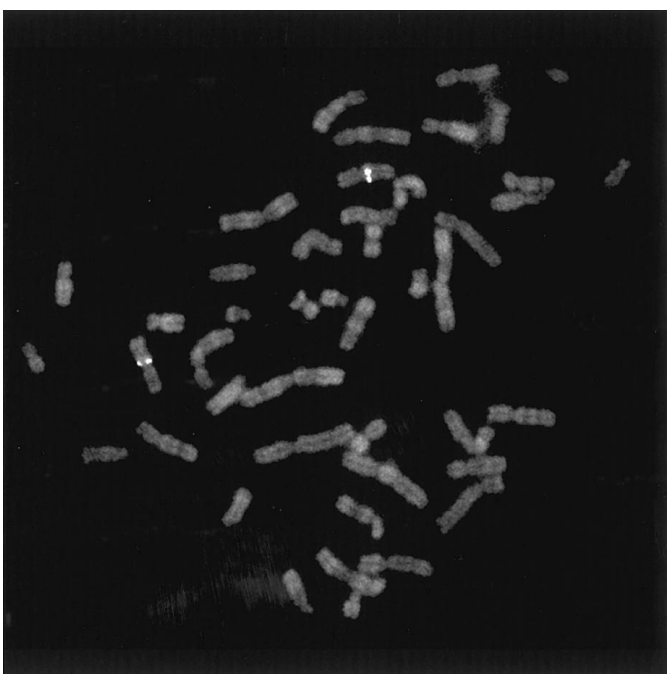

FIG. 2. FISH mapping of the DOC-1R gene. Metaphase chromosomes from human diploid cells were hybridized with labeled KB1574F2 BAC DNA. Typical twin-spot hybridization signals were observed on both of the two chromosome 11 at band q13 (bright spots).

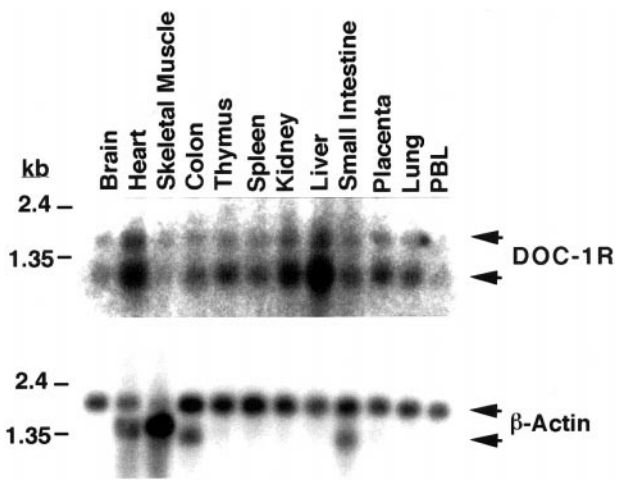

FIG. 3. Expression of DOC-1R mRNA in normal human tissues. A 12-lane multiple adult human tissue Northern blot was first hybridized with DOC-1R cDNA and then rehybridized with β-actin gene. RNA size markers in kb and tissues are indicated. PBL, peripheral blood leukocyte.

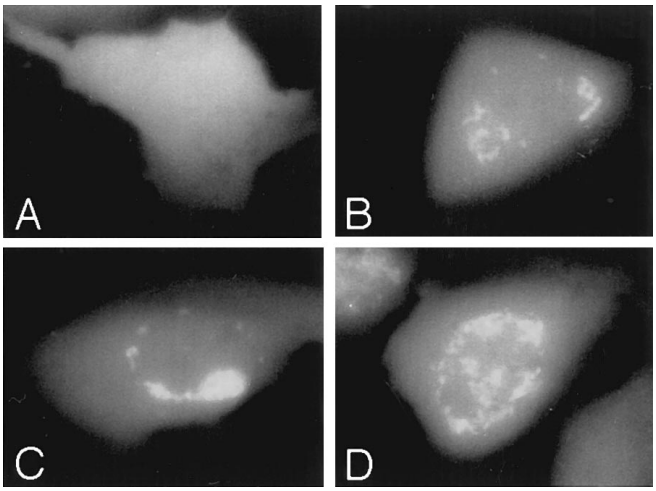

FIG. 4. Subcellular localization of GFP-DOC-1R fusion protein. U2OS cells were transfected with pEGFP-C3 control vector (A) and pEGFP-DOC-1R plasmid (B–D). Fluorescent signals were visualized after overnight incubation.

perinuclear (Fig. 4D) pattern. This heterogeneous distribution might reflect the protein trafficking process from cytoplasm to nucleus.

Since frequent LOH at chromosome 11q13 has been found in some cancers (9–12), and DOC-1R protein also has similar functional features with $p12^{DOC-1}$ in colony forming suppression (data not shown) and CDK2 association (Shintani and Wong, unpublished data), we performed mutation screening of the *DOC-1R* gene in 20 different cancer cell lines and 4 premalignant oral keratinocyte cell lines by RT-PCR-SSCP analysis. The *DOC-1R* coding region was amplified by RT-PCR using two primer pairs and the two overlapping fragments were then subjected to SSCP analysis. To increase mutation detection rate, we analyzed each fragment using duplicate SSCP gels with and without 5% glycerol (20). Although Fragment 1 showed variants in 2 ovarian cancer cell lines (Fig. 5, lanes 9 and 10), direct sequencing of these variants revealed a silent mutation at codon 60, Gln (CA<u>G</u> to CA<u>A</u>) (Fig. 6). Fragment 2 showed the same SSCP pattern in all cell lines examined compared to the normal controls. The failure to detect deleterious mutations in the DOC-1R gene may be attributed to the relative small cell line numbers examined or the technical limitations of PCR-SSCP analysis in mutation screening. However, our ability to detect a single base pair alteration argues that the approach we have selected is largely intact.

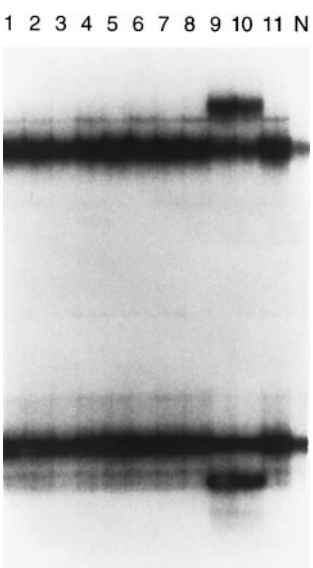

FIG. 5. RT-PCR-SSCP analysis. Total RNA from various cell lines were reverse transcribed, amplified using primers F1 and R1, and subjected to SSCP analysis. Lane N, normal oral keratinocyte cell line OKB6; lanes 1–11, various cancer cell lines; lane 9, ovarian cancer cell line Caov-3; and lane 10, ovarian cancer cell line SK-OV-3.

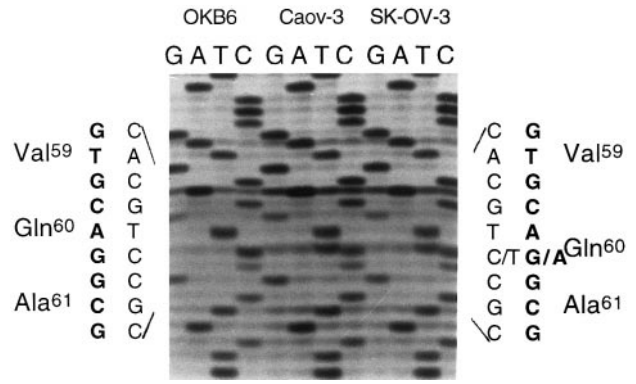

FIG. 6. Sequence analysis. PCR cycle sequencing of fragments exhibiting mobility shifts shown in Fig. 5 (lanes 9 and 10) reveals a silent mutation at codon 60, CAG to CAA.

We have identified a novel human DOC-1 growth suppressor related gene, *DOC-1R,* and mapped it to chromosome 11q13 region frequently affected by amplification or deletion in many types of human cancers. Although DOC-1R is an attractive tumor suppressor by position and early functional data, the lack of mutations in our preliminary mutation screen suggests that the gene is rarely involved in a select group of human cancers.

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