Down-regulation in Multiple Human Cancers of a Novel Gene, DMHC, from 17q25.1 That Encodes an Integral Membrane Protein

Iwao Mikami,1,2 Haruhito Harada,1 Hisaki Nagai,1 Michiko Tsuneizumi,1 Yukiko Nobe,1 Kiyoshi Koizumi,2 Sumio Sugano,1 Shigeo Tanaka2 and Mitsuru Emi1,4

1Department of Molecular Biology, Institute of Gerontology, Nippon Medical School, 1-396 Kosugi-cho, Nakahara-ku, Kawasaki-city 211-8533, 2Department of Surgery II, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8603 and 3Department of Virology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639

Frequent observations of allelic loss in chromosomal band 17q25.1 in a variety of human cancers have suggested that one or more tumor suppressor genes are present in that region. Moreover, a genetic locus for hereditary focal non-epidermolytic palmoplantar keratoderma, a condition associated with cancer of the esophagus (TOC; Tylosis with Oesophageal Cancer), lies in the same region. We screened cell lines derived from a variety of human cancers by reverse transcription-polymerase chain reaction (RT-PCR) to detect alterations in expression of genes within the region in question, by examining expressed sequence tags located there. These experiments identified an 1834-bp full-length cDNA encoding a novel, 441-amino acid integral membrane protein with seven putative transmembrane domains. This gene showed loss or extreme decrease of expression in 6 of 10 uterine cancer-cell lines, 2 of 11 hepatic cell carcinoma-cell lines, 2 of 7 lung cancer-cell lines, 1 of 6 gastric cancer-cell lines, and 1 of 10 breast cancer-cell lines. (We named it DMHC (“down-regulated in multiple human cancers”).) Our results suggest that loss of expression of DMHC at 17q25.1 may play an important role in development of variety of human cancers.

Key words: Tumor suppressor gene — Loss of heterozygosity — Chromosome 17q — TOC — Familial focal non-epidermolytic palmoplantar keratoderma

Human cancers derived from breast, esophagus, or ovary frequently show allelic losses on the long arm of chromosome 17,1-5) not only around the BRCA1 locus at 17q21, but also in chromosomal band 17q25.1. We recently defined a commonly deleted region in breast cancers within a 1-cM interval flanked by D17S1603 and D17S1839 on chromosome 17q25.1.1) Moreover, a genetic locus for hereditary focal non-epidermolytic palmoplantar keratoderma, a condition associated with cancer of the esophagus (TOC; Tylosis with Oesophageal Cancer), lies in an equally narrow, adjacent region flanked by D17S1839 and D17S785.6) On the basis of this evidence we hypothesized that the gene(s) mutated in germ-line DNAs of patients affected with the TOC syndrome may also be targets of allelic loss in sporadic tumors, and that one or more tumor suppressor genes are present in the 17q25.1 region.

We describe here the identification of a novel cDNA derived from chromosomal segment 17q25.1 that encodes an integral membrane protein with seven transmembrane domains. The full-length cDNA was 1834 bp long, and its putative product consisted of 441 amino acids. Reverse transcription-polymerase chain reaction (RT-PCR) experiments demonstrated loss or extreme decrease of expression of this gene in 12 of 54 cancer-cell lines derived from various tissues. As the gene showed loss of expression in multiple types of human cancers, we named it DMHC (“down-regulated in multiple human cancers”).

MATERIALS AND METHODS

Cloning strategy to identify genes with altered expression Forty expressed sequence tags (ESTs) from an interval on 17q25 flanked by D17S1352 and D17S785 on the human GeneMap 99 and Whitehead Institute Genome Database were obtained from Genome Systems and verified by sequencing. We examined these clones for altered expression in cancer-cell lines by the semi-quantitative RT-PCR method, using published primer sequences.

Cell culture In all, 54 cancer-cell lines were analyzed, including 10 derived from breast cancers (MDA-MB-453, CRL1500, YMB-1-E, MCF7, HBL100, OCUB-M, BT-20, BT-474, MDA-MB-435S, and SK-BR-3), 10 from uterine cancers (SIHA, HT-3, D98-AH-2, HeLaTG, HeLa, CaSki, ME-180, HeLaP3, HEC1-A, and SK-UT-1B), 11 from hepatic cell carcinomas (SK-Hep-1, C-HC-4, Hep-KANo CL-2, Hep-TABATA, HuH7, Hep G2, HT17, Li-7, PLC/PRF/5, Hep3B, and C3A), 7 from lung cancers (RERF-LC-AI, LU65, Lu99, PC-14, A549, EBC-1, and LK-2), 6 from thyroid cancers (WRO, NPA, 8305c, ARO, FRO, and 8505c), 4 from renal-cell cancers (OS-RC-2, VMRC-
RCW, RCC10RB, and Caki-1), and 6 from gastric cancers (HuGC-OOHRA, AZ521, H-111-TC, SH-10-TC, MKN-7, and NUGC-4). The cell lines were either donated by the Cell Resource Center for Biomedical Research at the Institute of Development, Aging and Cancer of Tohoku University or by Dr. Baba at Mie University, or purchased from the American Type Culture Collection (ATCC). Each cell line was cultured under the optimized conditions recommended by its respective distributor.

**DNA and RNA extraction and RT** DNA was extracted from all 54 cell lines by the phenol-chloroform procedures described previously; total RNA was extracted using chaotic reagent following procedures described elsewhere. mRNA from each cancer-cell line was prepared from $1 \times 10^8$ cells using the oligo-dT cellulose method (FastTrack 2.0 Kit, Invitrogen, Carlsbad, CA), following the manufacturer’s instructions. RTs were carried out with the SMART RACE cDNA Amplification Kit (CLONTECH, Palo Alto, CA) following the manufacturer’s instructions.

**PCR and RT-PCR** PCR amplifications were performed in 30-µl reaction volumes containing 10 pmol of each primer, 1× PCR buffer with 1.5 mM MgCl$_2$, and 0.5 unit of Taq DNA polymerase for 27 cycles, conditions that we empirically determined to enable comparison of gene-expression levels by ethidium-bromide staining after agarose gel electrophoresis. Thermo-cycle conditions for amplification were described previously. To quantify the expression levels of genes in each cancer-cell line relative to normal tissue, we performed duplex PCR experiments using expression of G3PDH as an internal control. The G3PDH primers were (forward) 5′-ACCACAGTCATGCCATCAC-3′, and (reverse) 5′-TCCACCACCCTGGTGCTGTA-3′. We carried out multiple pilot experiments to verify that 27 cycles of PCR is a suitable condition for quantitative comparison between control G3PDH gene and the tested gene, before carrying out our expression screening procedure.

**Northern-blot analysis** Membranes blotted with polyA RNAs from various normal human tissues were obtained from CLONTECH. We followed the technique described in the CLONTECH manual for northern-blot analysis. An EST fragment, SHGC-11589, was used to probe the blots. 5′ and 3′ rapid amplification of cDNA ends (RACE) 5′ and 3′ RACE reactions were performed on total cDNAs from normal human breast using the SMART RACE cDNA Amplification Kit (CLONTECH) according to the manufacturer’s protocol. Southern blots were performed to confirm that the longest 5′ and 3′ RACE products had been detected. The amplified products were cloned into TA cloning plasmid (AdvantAge PCR Cloning Kit, CLONTECH) and sequenced.

**Sequencing** The nucleotide sequence of DMHC represented by EST SHGC-11589 was determined by the BigDye (PE Biosystems, Foster, CA) Terminator cycle-sequencing method using an ABI PRISM 377 DNA Sequencer (PE Biosystems).

**Analysis of single-strand conformational polymorphism (SSCP) by silver staining** The coding region of DMHC cDNA was divided into 12 amplicons that overlapped their flanking segments by short sequences. Each segment was amplified by PCR; then about 10 ng of each PCR product was heat-denatured in the presence of 80% de-ionized formamide and electrophoresed in an 8% polyacrylamide gel, with or without 10% glycerol in 0.5× TBE, at 8 V/cm for 10 h at room temperature. DNA fragments were visualized by silver staining, as described previously.

**RESULTS**

**EST screening for down-regulated gene located on 17q25.1** A genomic map of microsatellite markers of 17q25.1 around the 1-Mb critical region that we recently defined as a commonly deleted region in breast cancer is...
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Fig. 2. Northern-blot analysis of expression of the gene represented by EST SHGC-11589 in various normal adult tissues. Each lane contains approximately 1 μg of polyA RNA.

Fig. 3. Nucleotide and predicted amino acid sequences of DMHC. Bold type indicates the coding region; protein sequence was deduced from the nucleotide sequence. The polyadenylation signal is shown by bold italic characters. Predicted transmembrane domains are underlined. Predicted cleavage site of the signal sequence is shown by an arrowhead.
presented in Fig. 1. The commonly deleted region of esophageal cancer and that of ovarian cancer and the TOC critical region are also displayed in the figure. Among 40 ESTs selected from an interval flanked by D17S1352 and D17S785 on 17q25.1, a region commonly deleted in breast, ovarian, and esophageal cancers, four candidate transcripts were identified, i.e., human counterpart of the 68-kd subunit of canine signal-recognition particles, human counterpart of murine ubiquitin-conjugating enzyme E2-230k, stSG2564 (GenBank accession No. Z395239), and SHGC-11589 (GenBank accession No. T55738). Among them, SHGC-11589 showed a loss or extreme decrease of expression in multiple cell lines derived from tumors that had arisen in a variety of tissues. To examine the normal expression pattern and size of the complete transcript, we hybridized the EST probe to a multiple-tissue northern blot, which revealed a transcript about 1.8 kb long that was expressed predominantly in liver, kidney, and skeletal muscle (Fig. 2).

**Cloning of full-length cDNA**

To clone the full-length cDNA for the gene corresponding to the SHGC-11589 sequence that was down-regulated in multiple cancer-cell lines, we used a full length-enriched cDNA library constructed by the Oligo-capping method using mRNA derived from colonic mucosa. In parallel, we performed 5′ and 3′ RACE experiments to extend the SHGC-11589 fragment. The cDNA-library screening yielded a positive clone, REC04315, that showed sequence identical to SHGC-11589 within the 3′ untranslated regions. Sequencing of the REC04315 clone revealed an 1834-bp insert (Fig. 3); the cDNA derived from RACE was 1807 bp long, encoding 441 amino acids, and its coding sequence was identical to that of REC04315. Since the sequence surrounding the initiation codon agrees with the Kozak rule, this ATG most likely represents the start site for translation.

**Structural features of the predicted protein**

Structural analysis using the TM pred and PSORT programs predicted that the putative DMHC product would contain a signal sequence of 23 amino acids and structural features of an integral membrane protein having seven transmem-
brane domains. These domains were predicted at the cDNA segments encoding, respectively, amino acid residues 52–74, 89–111, 122–144, 156–178, 202–224, 243–265, and 280–302 (Fig. 4). The nucleotide sequence exhibited homology with clones of Barstead Mus musculus cDNA (GenBank accession Nos. AA636372, AI510582, AI604030, AA122593, and AA636256).

Expression and sequence variation in cancer-cell lines

We examined expression of DMHC in 54 cancer-cell lines altogether. RT-PCR experiments indicated loss or extreme decrease of expression of this gene in 6 of 10 uterine cancer-cell lines, in 2 of 11 hepatic cell carcinoma-cell lines, in 2 of 7 lung cancer-cell lines, in 1 of 6 gastric cancer-cell lines, and in 1 of 10 breast cancer-cell lines. These results were confirmed by semi-quantitative RT-PCR using duplex-PCR method experiments, with the housekeeping gene G3PDH as an internal control. This analysis demonstrated loss or dramatic attenuation of expression of DMHC in many of the cell lines; for example see Fig. 5 for results of duplex-PCR in some of the hepatic cell carcinoma cells.

To search for sequence variations in DMHC we amplified the entire coding region of DMHC cDNA in the same panel of cancer-cell lines. The RT-PCR products were screened for variations using SSCP followed by visualization with silver staining. No sequence variation of DMHC coding region was detected in those specimens (data not shown).

DISCUSSION

We have described here the isolation, tissue expression, and down-regulation in cancer cells of a novel gene (DMHC) present in chromosomal region 17q25.1. The predicted product had structural features of a membrane protein with a signal peptide and seven transmembrane domains, according to analysis using the TM pred and PSORT programs. Based on these features, we speculate that the mature protein probably exists either on the cytoplasmic membrane or on membraneous structures (e.g., endoplasmic reticulum or Golgi apparatus) in the cytoplasm, and that it functions as a receptor for extracellular signals or as a channel for transporting small biological molecules across the membrane(s). The high degree of evolutionary conservation that the sequence exhibits across mammalian species (mouse and human) suggests that this gene plays a significant role in some aspect of extracellular or intracellular trafficking of biological signals/molecules.

The BRCA1 gene at 17q21 is responsible for predisposition to a hereditary breast/ovarian cancer syndrome. Also on the long arm of chromosome 17, TOC, a postulated gene linked to a hereditary disorder of keratinization that is frequently associated with esophageal cancer, lies in the interval between D17S1839 and D17S785 at 17q25.1.59 In multiple primary breast, esophageal, and ovarian cancers often show allelic losses on the long arm of chromosome 17 as well, and three or four distinct regions of deletion on 17q have been identified in sporadic breast cancers.2,16–19 We previously examined two of these loci in 178 primary breast cancers,21 and observed allelic losses at 17q25.1 in 97 tumors (55%). Losses were most frequent at markers around the TOC locus (48% at D17S1839 and 43% at D17S1603), where we identified a distinct commonly deleted region within a 1-cM interval. Therefore this region is a target of allelic losses in both breast and esophageal cancers, as well as being the inferred location for genetic mutations that confer predisposition to TOC. However, in spite of large-scale sequencing efforts around the TOC locus, aided by the Human Genome Project, no classical (class I category)20 tumor suppressor gene showing frequent mutations or deletions on both alleles in human cancers has been identified so far.

The present study demonstrated loss or extreme decrease of expression of DMHC located within the target region of allelic loss at 17q25.1, in multiple human cancer-cell lines. Genes whose expression is frequently lost in tumor genomes have been characterized as class II tumor suppressors.20 Our results suggest that the gene reported here might belong to class II because of its location in a commonly deleted region, its undetectable expression in numerous cancer-cell lines, and the absence of detectable mutations in those specimens.

It is tempting to speculate that regional inactivation of multiple class II tumor suppressors from this chromosomal band contributes to tumor formation. Possible candidate genes in the region include EVPL gene,21 which encodes a protein (enoplakin) belonging to a family of keratin-binding proteins; its alteration may be associated with keratinocyte dysfunction. Another candidate is the TK1 gene,22 which encodes a soluble thymidine kinase; its abnormal expression has been associated with several malignancies, including breast cancers. Detailed studies that include complete sequencing of genomic DNA in the region, functional analyses of DMHC through transfection to cancer cells or methylation analysis of its promoter region, should clarify how loss or extreme decrease of expression of this gene exerts an oncogenic effect in breast, esophageal, and/or ovarian tissues.

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