Identification of a Novel Gene, Selectively Up-Regulated in Human Carcinomas, Using the Differential Display Technique

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ABSTRACT

Using the differential display technique, selecting for genes up-regulated in renal cell carcinoma compared with normal renal parenchyma, we isolated a novel gene, designated DD96. As determined by in situ and Northern blot hybridization studies, DD96 is expressed only in rare normal epithelial cell populations, such as the proximal tubular epithelial cells of the kidney. However, it is expressed diffusely in malignant epithelial cells of the colon. In addition, DD96 is overexpressed in various human carcinomas originating from the colon, breast, and lung, as well as in a number of cell lines derived from tumors of these organs compared with normal epithelial cell populations. Furthermore, the expression of DD96 is induced in immortalized breast ductal epithelial cell lines compared with normal breast ductal epithelial cells, and, in vivo, in premalignant conditions, such as adenoma of the colon and ductal carcinoma in situ of the breast. Sequence analysis of a complete cDNA clone isolated from a human kidney cDNA library revealed that DD96 encodes a protein of approximately M, 13,500. These results suggest that DD96 may play a role in the early events associated with malignant transformation; however, its function remains to be determined.

INTRODUCTION

Many of the mechanisms involved in the development of cancer are unknown or poorly understood; however, it has become clear that successive genetic alterations play a crucial role in the development of malignant tumors (1–3). Therefore, it is increasingly important to identify the genetic changes that contribute to malignant transformation to gain a better understanding of the biology of oncogenesis. Such information may be expected to suggest new approaches for cancer diagnosis and management that will ultimately improve therapy.

Recently, various molecular methods and particularly differential screening techniques have provided valuable new approaches for the identification of genes that are selectively modulated in the course of oncogenesis (4–6).

We have used the recently described differential display technique (7–11) to identify genes that are up-regulated in a primary renal cell carcinoma compared with renal parenchyma from the same patient.

The expression of candidate genes selected on the basis of the differential display experiments was tested by in situ hybridization on various human carcinomas and corresponding normal tissues, as well as by Northern blotting on numerous cultured cell lines. Herein, we report the identification and cloning of a new epithelial specific gene (designated DD96) that is upregulated in various carcinomas and premalignant lesions compared with normal epithelial cell populations and certain immortalized epithelial cell lines. Our findings suggest that DD96 may play a role in the early steps of tumor progression.

MATERIALS AND METHODS

RNA and DNA Analysis. All studies were conducted with the approval of the Beth Israel Hospital Committee on Clinical Investigation (Boston, MA). Tissue samples were collected in the operating room, frozen in liquid nitrogen, and subsequently homogenized using a Polytron (Kinematica, Lucerne, Switzerland) in an extraction buffer containing 4.5 mM guanidine thiocyanate, 50 mM EDTA, 25 mM sodium citrate (pH 7.0), 0.1 mM 2-β-mercaptoethanol, and 2% sodium-N-sarcosine. Cultured cells were scraped from Petri dishes using a rubber policeman and homogenized in the same solution using a syringe equipped with a 20-gauge needle. RNA was purified by ultracentrifugation through a cushion of 5.7 M CsCl, as described previously (12). RNA samples to be used for differential display were further treated with RNase (Promega, Madison, WI) to remove genomic DNA contamination.

For Northern blot hybridization, RNAs (10 μg/lane) were denatured with formaldehyde, subjected to electrophoresis in 1% agarose formaldehyde gels containing 0.5 μg/ml of ethidium bromide, examined under UV light, and transferred to Biodyne filters (Pall Filter, Glen Cove, NY). After blotting, filters were UV cross-linked using a Stratalinken (Stratagene, LaJolla, CA). Northern blots were hybridized with the DNA probes isolated from the differential display experiments and labeled with [32P]dCTP (DuPont-NEN, Boston, MA) using a random primer-labeling kit from Amersham (Arlington Heights, IL). Control hybridizations were performed with a cDNA probe for the ribosome-associated protein 36B4 (13). High molecular weight genomic DNA was isolated from cultured mammary epithelial cells as described previously (14). DNA (10 μg) was digested with HindIII, size fractionated by electrophoresis, transferred to a nylon membrane, and hybridized with 32P-labeled DD96 cDNA probe.
**Differential Display.** Differential display reactions were performed using a Genehunter kit (Brookline, MA) according to the manufacturer’s protocol with a few minor modifications. In brief, 0.5 μg total RNA was reverse transcribed using T12MA, T12MC, T12MG, and T12MT primers. The same primers were used subsequently as 3’ primers to amplify the cDNAs by PCR along with 20 random 10-mer 5’ primers. Reactions were performed in PCR buffer containing 10 μCi 35S-labeled dATP (DuPont-NEN). The PCR products were displayed on a 6% sequencing gel to identify and select gene transcripts that were expressed differentially. The selected gene products were amplified and ligated into the pGEM-T vector (Promega) and sequenced using a Sequenase Version 2.0 DNA sequencing kit (United States Biochemical, Cleveland, OH). GeneBank was searched for sequence homology using the Hyperblast program.

**In Situ Hybridization.** In situ hybridization was performed on 6-μm fixed-frozen tissue sections using single-stranded antisense or control sense 35S-labeled riboprobes as described previously (15). These were generated after ligating the 230-bp DNA fragment DD96 isolated from the differential display into pGEM-T. A total of 28 tumors originating from the kidney (5 carcinomas), colon (4 carcinomas and 3 adenomas), lung (4 carcinomas), and breast (12 carcinomas) were examined. Of the 12, 7 infiltrating carcinomas of the breast contained areas of carcinoma in situ.

**Cells and Cell Cultures.** Normal mammary epithelial cells used in this study were obtained from reduction mammoplasty specimens as described (4). Normal urothelial cells were isolated from excess segments of donor human ureter from transplanted kidneys as described previously (16). Normal keratinocytes were obtained from Clonetics, Inc. (San Diego, CA). Human tumor cell lines SKMMC, HeLa, Jurkat E6, 293, UMRC, NCI-H747, SKMEL-5, H05, HT29, 184B5, T47D, ZR75-1, MDAMB231, MDAMB435, and MDAMB436 were obtained from American Type Culture Collection (Rockville, MD). The MCF7 cell line was a gift from Dr. M. Wicha (University of Michigan Cancer Center, Ann Arbor, MI). The MCF-10A cell line was a gift from Dr. S. Ethier (University of Michigan). RNAs for 21PT and 21MT-1 were gifts from Dr. V. Band (Tufts University, Medford, MA) and Dr. S. Ethier (University of Michigan), respectively. All cultured cells were maintained in DFCI-1 complete medium or in DMEM supplemented with 10% FCS (HyClone Laboratories Inc., Logan, UT) as described previously (4).

**cDNA Library Screening and DNA Sequencing.** A human kidney cDNA library was purchased from Clontech (Palo Alto, CA), and hybridized with the random, primed 32P-labeled 230-bp DNA fragment DD96 isolated from the differential display, as described in “Results.” A full-length cDNA clone was sequenced with oligonucleotide primers using the dideoxy-termination method (17).

**RESULTS**

**DD96 Isolation by Differential Display PCR.** Differential display reactions were performed to compare the pattern of gene expression of a renal cell carcinoma with that of normal renal parenchyma obtained from the same nephrectomy specimen. As shown in Fig. 1, many PCR products were found to be expressed in both the malignant and normal portions of the nephrectomy specimen, whereas other products were expressed in tumor or benign tissue only. Potential differentially expressed clones were selected for additional study, including in situ Northern blot hybridization. We identified a 230-bp cDNA clone, DD96, that was generated using T12MT as the 3’ primer and the AP-1 random primer 5’-AGCCAGCGA-3’ as the 5’ primer. DD96 was strongly overexpressed in the renal cell carcinoma compared with in the normal renal parenchyma. Among the clones selected by differential display, DD96 was the most consistently up-regulated gene in carcinomas compared with normal tissue samples and was, therefore, selected for more extensive studies.

**Cellular Localization of DD96 mRNA Synthesis by in Situ Hybridization.** Because both the malignant and normal tissues used for initial screening included a mixture of cell populations, in situ hybridization was used to determine the cellular source of DD96. Using an antisense RNA probe derived from the cloned cDNA fragment of DD96, we studied various human kidney, colon, lung, and breast tissue samples, as well as carcinomas arising from these organs (Figs. 2 and 3). In normal tissue samples, the level of expression of DD96 was extremely low, except in renal proximal tubular epithelium (Fig. 2A). Renal cell carcinomas showed a diffuse expression of DD96 of...
variable intensity in four (80%) of five of the cases studied: DD96 was expressed, although with varying intensity, by virtually all of the malignant cells (Fig. 2B).

In addition, both benign (adenomas) and malignant (adenocarcinomas) tumors arising in the colon strongly overexpressed, with signals of equal intensity, DD96 mRNA as compared with normal colonic mucosa (Fig. 2, C and D). Similarly,
Adenocarcinomas arising in the lung overexpressed DD96 mRNA in three (75%) of four cases studied as compared with normal lung (Fig. 2, E and F). In situ hybridization of normal breast tissue showed no significant labeling of ductal and lobular epithelia (Fig. 3A), whereas in situ hybridization of preinvasive ductal carcinoma showed an increase in labeling intensity compared with normal ductal and lobular epithelial cells (Fig. 3B). In situ hybridization studies of infiltrating carcinomas of the breast revealed great variations in the intensity of labeling, depending on tumor histology. Infiltrating ductal carcinomas showed a marked increase in labeling intensity (Fig. 3C), whereas infiltrating lobular carcinomas revealed no increased labeling intensity as compared with normal breast tissue (Fig. 3D). The labeling intensity was generally increased in infiltrating ductal carcinoma compared with ductal carcinoma in situ (Fig. 3, B and C).

In all cases studied, DD96 expression was confined to cells of epithelial origin; mesenchymal and inflammatory cells were not labeled detectably (Figs. 2 and 3). Control hybridizations using the corresponding sense cRNA probe were invariably negative on all tissues studied, whether benign or malignant.

Characterization of DD96 by Northern Blot Hybridization. Northern blots revealed that DD96 was not expressed detectably by a number of cultured nontumorigenic human epithelial cells, including those derived from normal breast epithelium, urothelium, and keratinocytes (Fig. 4). However, an approximately 1.0-kb mRNA transcript was detected in cultured cell lines derived from carcinomas of the kidney, colon, and lung (Fig. 4), as well as various cell lines derived from breast carcinomas (Fig. 5). Therefore, the pattern of expression of DD96 revealed by Northern blot analysis was consistent with the results obtained with in situ hybridization. It is of interest that expression of DD96 was also induced in two immortalized cell lines derived from normal breast ductal epithelium, namely, 184B5, immortalized after treatment with the carcinogen benzo(a)pyrene (18), and, to a lesser extent, in MCF10A, a spontaneously immortalized cell line (Ref. 19 Fig. 5).

To examine the possibility of gene amplification of DD96, genomic DNA was isolated from normal mammary epithelial and breast carcinoma cultured cells, digested with HindIII, and subjected to Southern blot analysis. A single band of approximately 12 kb was detected in all cell lines.

Isolation and Sequencing of a Full-Length DD96 cDNA Clone. Because the only normal adult tissue in which DD96 was expressed detectably was the proximal tubular epithelium of the kidney, we screened a kidney cDNA library to isolate cDNA clones containing the complete putative protein sequence. Several clones were isolated. The longest was 930 bp and contained a 114-amino acid open reading frame encoding for a protein of approximately Mr 13,500 (Fig. 6A). The sequence of the original DD96 fragment isolated by differential display matched the sequence of the 3’ end of the clones isolated from the cDNA library. A GeneBank search revealed partial
amino acid sequence homology between \textit{DD96} and the human calcium-transporting ATPase plasma membrane protein isoform 1B (20). In addition, it showed that the putative protein sequence contained two potential phosphonylation sites, a markedly hydrophobic amino terminus, with a possible signal peptide, and a hydrophilic carboxyl terminus (Fig. 6B). Our clone has been submitted to the GeneBank, where it is accession number U21049.

**DISCUSSION**

Malignant transformation is a multistep process that is likely to involve a large number of genetic alterations. Studies conducted over the past decade using various approaches have identified numerous genetic changes associated with malignancy, notably oncogenes and tumor suppressor genes. Nonetheless, in the vast majority of cases, the genetic changes leading to cancer are still unknown. The development of differential
screening techniques has provided new tools, which can be used to identify genes associated with tumorigenesis (4–6). One of these techniques, the recently described differential display (7, 9), is more efficient and sensitive than are most other such methods. We have used this technique to isolate and begin to characterize a new gene, DD96, which is expressed in the proximal tubular epithelium of the normal adult kidney and in many cancers of epithelial origin arising from kidney, colon, lung, and breast. The overexpression of DD96 in renal cell carcinoma may reflect, at least in part, that most renal cell carcinomas are derived from proximal tubular epithelium (3). DD96 seems to be an epithelium-specific gene encoding for a protein of approximately M, 13,500.

Although, at present, the function of the protein encoded by DD96 is not known, it is up-regulated at early stages of tumor progression, as determined by the overexpression of its mRNA in benign colonic adenomas and by carcinoma in situ arising in breast. Studies of nontumorigenic cultured cells have demonstrated an association between its overexpression and cell immortalization. Cellular immortalization and transformation are two of the most important events leading to a malignant phenotype (3, 21). Immortalization can be induced by several agents, including oncogenes and carcinogens. Among them are the adenovirus early region 1A and the polyoma large T antigen, as well as others (21). Immortalized cells become more sensitive to additional genetic events, resulting in the transformed phenotype. Apart from oncogenes, relatively few genes have been linked to the immortalization process, and the cellular mechanisms leading to immortalization are far from being understood fully. The gene described herein, DD96, may play a role in the biological events leading to immortalization and, perhaps, in the early steps of cancer development. The role played by DD96 in that process remains to be determined, and several experiments are presently being performed to determine its function. The presence of hydrophobic regions suggests that this protein may be membrane associated. The restrictive pattern of expression of DD96 in normal tissues to renal proximal epithelial cells and its partial amino acid sequence homology to a calcium-transporting protein (20) raise the possibility that it may be involved in transport mechanisms; however, this remains to be demonstrated.

Carcinogens have been implicated in the development of breast cancer, as well as other cancers (21, 22). It is of interest that one of the immortalized breast ductal cell lines, 184B5, was immortalized after exposure to the carcinogen benzo(a)pyrene (18).

An important feature of our work has been the direct application of differential display to human tumors and normal adult tissues, as compared with the more usual approach of screening cultured cell lines. The direct approach we have taken has been avoided by others because it is recognized that mammalian tissues are composed of complex mixtures of parenchymal and stromal cells; therefore, a gene or genes identified by differential display methodologies and found to be overexpressed or underexpressed by Northern blot analysis may just as easily be the product of benign host cells as of tumor cells. To settle the question of cellular origin, we made use of in situ hybridization, a powerful technique for localizing mRNA synthesis to specific cells. The results obtained indicate that our direct approach is feasible and opens the way for direct comparative analysis of other genes expressed differentially by tumors and normal tissues. Because the relationship of cultured cell lines to authentic human tumors is always questionable, we believe that this approach should come to enjoy broad application.

This article reports the identification and cloning of a new epithelial cell-specific gene up-regulated in immortalized and tumor cell lines in vitro and in premalignant and malignant tumors in vivo. The determination of the function of this protein could provide additional information about the mechanisms involved in human carcinogenesis.

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