Identification of a Gene Containing Zinc-Finger Motifs Based on Lost Expression in Malignantly Transformed Rat Ovarian Surface Epithelial Cells

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ABSTRACT

We have used a rat model of epithelial ovarian cancer to identify a gene that shows decreased or lost expression in five of eight independently transformed rat ovarian surface epithelial cell lines compared to the normal progenitor cells. Hence, we refer to this gene as Lot1 (lost on transformation 1; GenBank accession no. U72620). The most abundant transcript of the gene is ~6 kb. This sequence contains a 1749-nucleotide open reading frame and, within the 3' untranslated region, 22 near-perfect 60-70-bp repeats and adenine- and uracil-rich areas. The deduced amino acid sequence from the open reading frame contains seven zinc-finger motifs of the C2H2 type, as well as proline-, glutamine-, and glutamic acid-rich areas. The gene maps to the short arm of chromosome one in the rat. Lot1 shows a limited distribution of expression in normal rat tissues, including ovary, which shows abundant expression. Furthermore, examination of DNA derived from multiple species indicates that the gene is widely conserved.

INTRODUCTION

Several features of malignant ovarian tumors complicate determining the molecular genetic basis for their initiation and progression. It is accepted that the vast majority of these tumors arise from the ovarian surface epithelium (1). In contrast to colon cancer, in which a well-defined series of morphologically identifiable precursor lesions occur, there is substantial controversy as to whether benign ovarian tumors, changes in the growth pattern of the surface epithelium, or tumors of low malignant potential are precursors of overt ovarian malignancies (2, 3). Furthermore, the symptoms of ovarian cancer are vague, and the location of the ovaries precludes invasive screening procedures such as can be performed on the colon by endoscopy. Hence, the majority of ovarian tumors available for molecular genetic analysis are from patients with late-stage disease. These specimens likely contain a combination of causal and random genetic changes. At a minimum, the number of abnormalities is large, as is manifested by cytogenetic analysis and allotyping studies (4, 5). The latter indicate frequent loss of heterozygosity on many different chromosomes, which has complicated selection of chromosomal areas from which to initiate deletion mapping and positional cloning efforts to identify ovarian cancer genes.

Here, we present an alternative approach to obtain clues to genes involved in the etiology and progression of ovarian cancer. We have developed a rat model of ovarian cancer (6, 7) in which repeated requirement for growth of surface epithelial cells in vitro results in malignant transformation. This is consistent with the postulated role in human ovarian cancer of repeated wound repair/growth by the surface epithelium at the site of ovulation, as is evidenced by the increased ovarian cancer risk of nulliparous women. Using a method referred to as differential display (8–10) and this tissue culture model of ovarian cancer (6, 7), we compared gene expression in normal and spontaneously transformed rat ovarian surface epithelial cells. We identified several transcripts that were expressed at different levels in transformed rat ovarian surface epithelial cells as compared to their normal cellular counterparts. One cDNA clone showed loss of expression in two independent malignantly transformed cell lines. This clone provided a starting point for construction of a composite cDNA derived from a gene we designate Lot1 (GenBank accession no. U72620). These reagents allowed us to determine tissue distribution of expression, loss of expression in other transformed rat ovarian surface epithelial cell lines, and interspecies conservation and to map the chromosomal location.

We present evidence that Lot1 is a previously unidentified zinc-finger motif containing protein. It is noteworthy that among the diverse proteins containing zinc-finger motifs, many have roles in oncogenesis and/or growth regulation. Perhaps the best example is the WT1 gene, which contains four zinc fingers and functions as a transcription repressor. The inactivation of both alleles of WT1 has been shown to be responsible for the development of some cases of Wilms' tumor (nephroblastoma), one of the more common pediatric malignancies (11). This role of lost expression in oncogenesis places the WT1 transcription factor in the category of cancer genes referred to as tumor suppressor genes (5, 12). Our data suggest that Lot1 may also belong to this category of genes.

MATERIALS AND METHODS

Cells and Cell Culture. Rat ovarian surface epithelial cells were obtained from the ovaries of adult female Fisher rats by selective trypsinization (6, 7). The cells were grown in vitro as described previously (6, 7) using DMEM and 4% fetal bovine serum. The cells in early passage, i.e., four to six passages, were considered normal. After 30–40 population doublings, individual cell lines frequently developed characteristics of malignant cells including the capacity to form tumors (6, 7, 13). As such, it was possible to examine gene expression differences between early-passage "normal" cells, transformed cells that gave rise to tumors, and cell lines established from those tumors (13).

Differential Display. The technique of differential display allowed individual segments of mRNAs to be visualized and a comparison made of their presence and relative abundance between normal and transformed rat ovarian surface epithelial cells. This was accomplished using the PCR to amplify sequences delineated by pairs of oligonucleotide primers: one primer was oligodeoxycytidine (so that it would anneal to the polyadenylate tail of mRNAs) and the other was short and arbitrary in sequence (so that it would anneal at different positions relative to the oligo(dT) primer. Refs. 8 and 10). The PCR products were separated by PAGE and visualized by autoradiography (Fig. 1). An Individual cDNA band that showed a consistent pattern of alteration was cut from the dried gel using the autoradiogram as a guide to its location, eluted, and reamplified as described previously (9, 10). The reamplified DNA fragments were cloned into the pcRII plasmid vector using TA-cloning (Invitrogen, San Diego, CA) to create a small cDNA library. A member of this library, P2–2, was identified based on its use as a probe on a

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3 The abbreviations used are: Lot1, lost on transformation 1; cLot1, rat Lot1; WT1, Wilms' tumor 1; FISH, fluorescence in situ hybridization; EGR, early growth response.
Northern blot to yield a pattern of expression consistent with that seen on the differential display autoradiograph.

Construction of cdNA Libraries, Screening, and Analysis of Clones. A whole rat ovary cdNA library was constructed in AZAPI vector (Stratagene, La Jolla, CA) as described previously (13). This library (1 x 10^8 recombinant plaques) was screened using the 146-bp fragment (Fig. 2, nucleotides 4204—4350) obtained by differential display. A cdNA designated as RO21 (2071 bp; primer is shown at the bottom of the figure). The 5’ primer is shown at the bottom of the figure.

RESULTS

Detection of a Transcript Present in Decreased Abundance in Transformed As Compared to Normal Rat Ovarian Surface Epithelial Cells. We have developed a series of spontaneously transformed rat ovarian surface epithelial cell lines (6, 7). These cell lines, along with the normal cells from which they arose, provide a model system in which the consistency of genetic changes associated with independent induction of the malignant phenotype can be examined in a defined genetic background. Malignant cells and cells from the tumors they produced were examined for apparent differences in gene expression as compared to their normal progenitor cells using the differential display technique (8—10). Fig. 1 shows an autoradiograph from a representative experiment. Numerous band differences were observed between tumor cells, the transformed cells that yielded the tumors, and normal rat ovarian surface epithelial cells. In some cases, consistent apparent decreased gene expression was seen in independently transformed cells compared to their normal progenitors. Such consistent changes served as the basis for further study. The analysis template, 60 μM of dNTPs, 1× PCR buffer, 1 mm primer, 5% DMSO, and 2.5 units of AmpliTaq DNA Polymerase (Perkin-Elmer Corp.). The cycling conditions consisted of 94°C for 5 min, 19 cycles of 94°C for 5 s, 65—0.5°C/cycle for 30 s, and 72°C for 1 min, followed by an additional 25 cycles of 94°C for 5 s, 55°C for 30 s, and 72°C for 1 min, and then extending at 72°C for 5 min in a PTC-100 Thermal Cycler (M. J. Research, Inc., Watertown, MA). PCR products were cloned into the pGEM-T vector (Promega, Madison, WI). Purified plasmid preparations were treated with the SacII-SpeI restriction endonucleases. The released inserts were separated from the plasmid by agarose gel electrophoresis and were purified from the agarose gels using either the Nal/Glass Bead technique or Centricon concentrators (Amicon, Beverly, MA). Labeling was by random priming (Prime-IT II, Stratagene).

RNA and DNA Analysis. Total RNA generally was isolated from tissues and cell lines by the single-step guanidinium isothiocyanate/phenol extraction method (15, 16). In the case of liver, extraction was performed with an additional LKI treatment. Total RNA (15 μg/lane) was separated on 1% agarose gels containing 2.2 μm formaldehyde. Transfer was to Nylon membranes (Micron Separations, Inc.) by capillary action. The membranes were hybridized with [α-32P] dATP-labeled (DuPont NEN, Boston, MA) cdNA probes by random priming. Prehybridization was performed at 42°C in buffer containing 50% formamide, 4× SSC, 4× Denhardt’s solution, 0.4 M NaH2PO4 pH 6.5, 0.83% glycerine, 160 μg/ml salmon sperm DNA, and 6.83% SDS adjusted to pH 7.4. The hybridization solution contained 50% formamide, 3× SSC, 50 M NaH2PO4, 112 μg/ml salmon sperm DNA, 1% SDS, 1.1× SSC, 1% dextran sulfate adjusted to pH 7.4. The membranes were washed for 1 h at 65°C in 2× SSC, 0.5× SET (10% SDS, 0.05 M EDTA), 0.1 M Tris-base, pH 7.5 (solution A) and for 1 h at 55°C in 0.1× SSC, 0.5× SET, 0.1% NA PP, pH 8 (solution B). Visualization was by autoradiography (X-OMAT, Kodak, Rochester, NY). Southern blot hybridization and cdNA library screening were done at 65°C in Church buffer (0.5 M NaPO4, pH 7.1, 2 mM EDTA, 10% SDS, 0.1% NA PP). The blots were washed in solution A at 65°C for 1 h and solution B at 60—65°C for 1 h. Visualization was as for Northern blots.

FISH. Metaphase spreads were prepared by standard procedures (7). The RO21 cdNA clone was labeled with biotin 16-dUTP using a nick translation kit (Oncor). FISH and detection of immunofluorescence were performed basically according to the technique of Pinkel et al. (17). The chromosome preparations were counter stained with 4',6'-diamidino-2-phenylindole in antifade solution (Oncor). Metaphase spreads were observed using a Zeiss Axiophot microscope and images were captured by a cooled CCD camera connected to a computer workstation. Digitized images of 4',6'-diamidino-2-phenylindole staining and fluorescent signals were merged as described elsewhere (18, 19). By the hybridization and washing conditions used, examination of normal rat metaphase spreads for the localization of a single copy gene routinely yields only fluorescent signals at the normal locus of the gene on one or both homologues, often as a pair of dots (one on each chromatid). Nonspecific (background) hybridization typically is very low (i.e., usually less than two signals per metaphase; Refs. 19—21).

POLYMERASE CHAIN REACTION AMPLIFICATION AND GENERATION OF PROBES. The amplification reactions were carried out in a final volume of 40 μl and contained 100 ng of

OVARIAN CANCER GENE

Fig. 1. Autoradiograph of differential display gel of cell line (CL) triplets 12 and 14: E, early passage; L, late passage transformed; T, cells from the tumors produced by transformed cells. Arrows, banding differences between phenotypically normal and transformed cells. Top arrows, loss of a band in both transformed cell line 12 and 14. Bottom arrows, similar loss of a band in both transformed cell lines. *, source of material used to identify the 146-bp cdNA (P2—2; see “Results”). T12MA, T12MG, T12MC, T12MT. 5′ primer = 5′-CTTTCTACCC
of one such change (Fig. 1, *) serves as the basis of the work presented here. The PCR products present in the region of the gel that gave rise to the band that is visualized in normal (i.e., early passage) cell line 12 but not in its malignant counterpart were reamplified, and a 46-bp cDNA fragment was used as a probe to screen a whole rat ovary.

The near-perfect tandem repeats (—60—70 bases, single underlined with vertical marks on the line to delineate individual repeats) beginning at base 2970 with the sequence @ TTGT I'GAGA.

The RNA species detected was approximately 6 kb in size (data not shown). Sequence analysis of the 146-bp cDNA (designated P2—2) revealed that it was not part of a known gene.

**Isolation and Characterization of a Candidate cDNA.** The 146-bp cDNA fragment was used as a probe to screen a whole rat ovary cDNA library. This process yielded a clone —2 kb in size containing 22 near-perfect 60—70-bp repeats (Fig. 2), no open reading frame of substantial size, and no poly(A) tail. Examination of a multiprobe Southern blot with this clone as a probe suggested that a homologous gene might exist in mouse and yeast (data not shown). This probe was also used to examine the pattern of **Lotl** expression in normal rat tissues, immortal nontumorigenic rat surface epithelial cell lines, and eight malignantly

Fig. 2. Nucleotide and deduced amino acid sequence of the **Lotl** cDNA. The open reading frame and near-full-length nucleotide sequences of rat **Lotl** are shown. Nucleotide and deduced amino acid sequences are numbered from the 5' terminus.

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procedures was used to obtain a near-full-length cDNA (Fig. 2). Genetic material as the mechanism of lost expression (data not shown). Blots with Lot1 cDNA probes, however, did not suggest loss of transcript. Examination of Southern blots revealed that DNA from all species examined likely contains a closely related gene (Fig. 7).

DISCUSSION

A number of conclusions may be drawn from the work presented. The most tantalizing possibility is that the strategy of using growth stress-induced malignant transformation of rat ovarian surface epithelial cells as a starting point may identify genes of relevance to the etiology and progression of human ovarian cancer; however, validation must await the cloning of the human homologue, mapping of its chromosomal location, and analysis of human ovarian cancers. Many facets of the model and the data presented, however, favor this interpretation.

Current evidence suggests that the development of malignancy in transformed (tumorigenic) rat ovarian surface epithelial cell lines. Northern blot analysis (15 μg total RNA/lane) was accomplished by hybridization with 32P-labeled EcoRI—NotI restriction fragment of the RO21 clone. Expression in normal rat ovarian surface epithelial cells (five early-passage cell lines) is compared to expression in eight tumor cell lines (Nutu) derived from independent malignantly transformed rat ovarian surface epithelial cells. Loading equivalency is shown by hybridization with a glyceraldehyde phosphate dehydrogenase (GAPDH) probe and by ethidium staining (Total RNA). Migraton of 18S and 28S RNA is also shown.

open reading frame of 1749 nucleotides, and 2729 bp of 3'-untranslated sequence. rLot1 encodes a predicted protein of 583 amino acids with a molecular weight of approximately 66,000 (Fig. 2). Analysis of the deduced amino acid sequence by the programs BLAST Enhanced Alignment Utility and MOTIF/Genetics Computer Group revealed that the protein contained seven zinc-finger motifs. The relationship of the individual fingers to one another and to the zinc-finger motifs of known proteins is shown in Fig. 5. The gene was mapped by FISH to the short arm of rat chromosome 1 (Fig. 6), and hybridization of a probe derived from a clone encompassing bases 1065—2865 (Fig. 2) to a multispecies Southern blot revealed that DNA from all species examined likely contains a closely related gene (Fig. 7).

Fig. 3. Northern blot analysis showing expression of LOT1 in rat tissue RNA. Total cellular RNA was isolated from various tissues of 14-week-old Fisher 344 rats. Total RNA (15 μg/lane) was denatured, separated by electrophoresis on an agarose (1% agarose—2.2 M formaldehyde) gel, and blotted. Hybridization of blot was with a 32P-labeled probe encompassing bases 2315—4386 of the Lot1 cDNA (Fig. 2).

Fig. 4. Loss of Lot1 expression in malignantly transformed rat ovarian surface epithelial cell lines. Northern blot analysis (15 μg total RNA/lane) was accomplished by hybridization with 32P-labeled EcoRI—NotI restriction fragment of the RO21 clone. Expression in normal rat ovarian surface epithelial cells (five early-passage cell lines) is compared to expression in eight tumor cell lines (Nutu) derived from independent malignantly transformed rat ovarian surface epithelial cells. Loading equivalency is shown by hybridization with a glyceraldehyde phosphate dehydrogenase (GAPDH) probe and by ethidium staining (Total RNA). Migraton of 18S and 28S RNA is also shown.

Fig. 5. Alignment of zinc-finger region in LOT1 protein. A, comparison of Cys- and His-rich repeats of the seven zinc fingers (ZINCF) derived from the full-length amino acid sequence of rLOT1 to the zinc fingers of other proteins. The alignment shows the EGR sequence of the rLOT1 protein as shown in Fig. 2. B, homology of deduced amino acid sequences of the Cys-zinc finger motifs. The relationship of the individual fingers to one another and to the zinc-finger motifs of known proteins is shown in Fig. 5. The gene was mapped by FISH to the short arm of rat chromosome 1 (Fig. 6), and hybridization of a probe derived from a clone encompassing bases 1065—2865 (Fig. 2) to a multispecies Southern blot revealed that DNA from all species examined likely contains a closely related gene (Fig. 7).

Fig. 6. Digitized, computer-generated image of FISH with fluorescein-labeled Lot1 probe to a normal rat metaphase spread. White signals were seen against the gray chromosomes. In this metaphase, two signals are seen on the short arm of one copy of chromosome 1 and one signal on the other copy (arrows).
different tissues, at least in part, follows divergent genetic pathways. The rat model system described uses ovarian surface epithelial cells, and this is the cell type in the human from which most malignant tumors of the ovary arise (1). Efforts to induce malignant transformation in ovarian surface epithelial cells is of particular interest because there has been substantial speculation as to why they are the ovarian cells most likely to undergo malignant transformation. Epidemiological data show a markedly increased risk of ovarian cancer in nulliparous women and women who have used fertility drugs (22–24). This observation has fueled speculation that the driving force for this latter possibility is provided by a potential alternative polyadenylation signal (AAGAAA; bases 2587–2592) upstream of the first poly(A) signal of Lot1 in other independently transformed rat ovarian surface epithelial cell lines was seen at high frequency, further implying the possibility of an important causal or contributory role. We were hopeful that the chromosomal location of the rat gene could be used to suggest the chromosomal location of the human homologue based on regions of synteny between the species. This could then provide additional circumstantial evidence of significance if the gene resided in a human disease locus. Unfortunately, Lot1 is the only gene thus far mapped to the short arm of rat chromosome 1. Thus, speculation as to the location and potential role of LOT1 in human disease was not possible based on our data in the rat. This observation has led us to speculate that the driving force for oncogenesis of the ovary in situ.

The predicted amino acid sequence of LOT1 revealed seven classical zinc-finger domains in a tandem repeat beginning in the NH2-terminal region of the protein. A data base search indicates that the polypeptide belongs to the C2H2 class of zinc-finger proteins, typified by the Xenopus laevis 5S RNA TIIIF (30, 31), which has a sequence of C-X5–7-C-X12–17-H-X4–5-H. A sequence comparison with one of the zinc-finger modules, including the preceding linker residues, indicates that this region of the Lot1 protein is homologous to proteins encoded by the EGR genes and the WTI1 gene, and is evidenced by the consensus sequences extensively shared among them (see Fig. 5). These and several other proteins of the C2H2 zinc-finger family exhibit developmental and tissue-specific expression in mammals (32) and are most often DNA binding proteins of the transcription factor class. The function and role of the Lot1 protein in the mammalian developmental processes, however, remains to be determined.

Additional features of the translated mRNA of interest are its high proline and glutamine content. Proline-rich regions have also been found in several DNA-binding proteins, including EGR2/Krox-20 (33), Krippel gene product (34), and WTI1 (35). These specific domains may either repress gene transcription, as is observed in WTI1 (36), or activate transcription of other genes (34, 37–39). The COOH-terminal half of the amino acid sequence of Lot1 is also rich in glutamine. Glutamine-rich domains have been found in the Sp1 transcription factor and are believed to represent a motif for transcriptional activation (40, 41). Taken together, the structural features discussed above lead us to speculate that the Lot1 protein is a DNA binding protein that could play a role as a transcriptional regulator.

Northern blotting results and sequence analysis suggest that more than one mechanism may control the expression of the Lot1 gene. Hybridization data show different transcript sizes with near-identical patterns and ratios of expression. This may be due to alternative mRNA processing events [i.e., formation of splice isoforms, as is also found in some genes, including WTI1 (42)] and/or alternative polyadenylation signals. Support for this latter possibility is provided by a potential alternative polyadenylation signal (AAGAAA; bases 2587–2592) upsteam of the first poly(A) stretch. Functional analysis of the truncated proteins that could be encoded by these shorter transcripts will be of interest.

An additional feature within the 3′-untranslated region of the rat cDNA is of interest. This region contains AU-rich areas, including AUUAU, AUAU and AUAAU (non-AUUAU) elements. Such sequences have been shown to markedly decrease stability when they are present in the 3′-untranslated regions of many labile mammalian mRNAs, such as those that encode cytokines and proto-oncogenes (43–46). It will be interesting to determine whether this particular level of posttranscriptional modification is responsible, either fully or partially, for the lost or decreased Lot1 expression in malignant ovarian surface epithelial cells.
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