Identification of a Gene Encoding a Human Oxysterol-binding Protein-Homologue: A Potential General Molecular Marker for Blood Dissemination of Solid Tumors

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ABSTRACT

This study describes a new potential role in human cancer for a gene, HLM, isolated by differential display, that bears homology to an oxysterol-binding protein. A significant association between increased expression of HLM with metastatic disease was found. HLM mRNA levels were increased in circulating tumor cells in patients’ peripheral blood and in primary human epithelial cells expressing the human papillomavirus16 E6 and E7 proteins. HLM mRNA was not detected in most normal human tissues, including peripheral blood and lymph node. These findings indicate that HLM may function as a potential marker for tumor dissemination.

INTRODUCTION

Lung cancer is a leading cause of cancer-associated deaths in the United States, followed by colorectal and breast cancer. Mortality associated with these cancers is often a consequence of metastasis of the cancer cells to secondary sites. As cancers progress, tumor cells can develop the capability of invasion and/or metastasis. The formation of metastases from a variety of tumor types share common steps that are independent of the primary tumor location and necessarily involves tumor dissemination by the entry of cells from the primary tumor into the peripheral blood circulation. Although the relationship between circulating tumor cells and the development of secondary disease is not fully understood, it may be possible to detect premortality of tissues by identifying tumor markers in blood samples.

A useful general marker for tumor dissemination has not yet been described. Many tissue-specific markers have been identified (1) but a few potential general cancer markers such as cytoketatin 19 (CK19), carcinoembryonic antigen (CEA), keratin 19 (K19), cytokines, and telomerase have been found to be differentially regulated in a variety of human cancers (1–3). However, most of these markers were also detected in normal peripheral blood, bone marrow, or lymph nodes, rendering them unsuitable for use as global tumor dissemination or metastasis markers (1, 4–6).

OXYB2 is a cytosolic protein that binds a range of oxysterols, the binding affinities of which generally correspond to potencies in regulating sterol metabolism, which suggests that this protein may mediate the regulatory actions of oxysterols (7). It is believed that OXYB transports sterols from lysosomes to the nucleus, where the sterol down-regulates the genes for the LDL receptor, HMG-CoA reductase, and HMG synthetase (8). The first human OXYB cDNA cloned and sequenced is located on 11q (8).

We address the application of HLM, a human OXYB-homologue mRNA isolated by DD, as a general molecular marker for detection in the peripheral blood of metastatic tumor cells derived from solid tumors. The low expression or absence of HLM mRNA in peripheral blood leukocytes and lymph nodes as well as in most normal human tissues and its up-regulation in several cancer cell lines, breast cancer tissues, and lung cancer blood samples suggests it as a general marker for systemic spread of tumor cells. The detection of small numbers of such cells may provide a tool with which to evaluate metastatic progression.

MATERIALS AND METHODS

Cell Lines and Culture. The cell lines were maintained in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum at 37°C and 5% CO2.

Patients Samples. A total of 21 tissue samples from breast cancer patients and 13 blood samples from patients with histologically documented nonneumatological cancer, with either localized or metastatic disease, were analyzed. All of the lung cancer blood samples analyzed in this study were collected before antitumor treatment or surgery. For negative controls, nine blood samples from healthy donors with nonneoplastic disease and one normal adjacent breast tissue were included in this study. The Scientific Committee of University Hospital-Universidade Federal do Rio de Janeiro, Brazil and Dana-Farber Cancer Institute (Boston, MA) approved this investigation.

RNA Preparation. Venous blood (3 ml) was obtained with a standard venipuncture technique using anticoagulant. Whole blood was centrifuged at 1800 × g for 40 min in a clinical centrifuge. The cells present in theuffy coat were collected and washed with 1 ml of buffer containing 10 mM Tris-HCl (pH 7.6), 5 mM MgCl2, and 10 mM NaCl. They were centrifuged at 1800 × g for 1 min, and this step was repeated 3 times. The pellet containing nucleated cells was incubated 5 min with 1 ml of Holmes Boner buffer (9). RNA was extracted with phenol-chloroform (1:1; pH 6.0; Ref. 9). The pellet containing RNA was resuspended in 300 μl of sterile water, and DNase I was treated in TE buffer (pH 7.5) containing 100 mM MgCl2 and 10 mM DTT, and 40 units of RNase inhibitor (10). Total cytoplasmatic RNA from exponentially growing cell cultures was isolated using the Trizol reagent as described (Life Technologies, Inc., Gaithersburg, MD). RNA preparation of tumor biopsies was conducted as described previously (11).

Cell Spiking and DD. Cell spiking experiments were used to test the detection limit of RT-PCR of tumor cells in blood. Known numbers of HeLa cells were added to 3 ml of whole blood before buffy coat separation. RT-PCR and PCR amplification were performed with 2 μM primers (HT11C and HAP28) provided by RNA Image kit per the manufacturer’s instructions (Gene Hunter Corporation, Nashville, TN). PCR-amplified cDNA products were resolved on a 6% DNA sequencing gel (Genomyx Corporation, Foster City, CA). The bands of interest were excised from the gel, isolated, and reamplified by PCR (12, 13).

Direct Sequencing. The nucleotide sequence for HLM PCR fragments was determined by the dideoxy termination method (14), using the Circumvent Sequencing kit (New England BioLabs, Beverly, MA) with [γ-32P]ATP 5’ end-labeled primers, as described in the supplier’s instructions. The DNA template was purified from an agarose gel (QIAquick). The samples were run on a 6% polyacrylamide gel (Genomyx Corporation) at 60°C, 3000 V, 125W, and sequenced using Ampli Taq DNA polymerase, FS dye-terminator, modified from Applied Biosystems (Molecular Biology Core Facility, Dana-Farber Cancer Institute, Boston, MA).
RT-PCR Amplification and Northern Blot. The RT reaction was performed with Superscript II per the manufacture’s instructions (Life Technologies, Inc.). The HLM gene was amplified using primers 5'-AAAAATGATTGAANCCGGGTGACCT-3' and 5'-TTATTAAGGTACAAAGGGTCTCGC-3'. PCR reactions were performed with 2.5 units/μl AmpliTaq (PerkinElmer, Branchburg, NJ), 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 1.5 mM MgCl₂, and 200 μM of each dNTP and 50 μM of each primer in 50-μl reaction mix. The PCR reaction was programmed as follows: initial denaturation at 94°C for 5 min, then at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min for 35 cycles; elongation at 72°C for 5 min; and refrigeration at 4°C. Radioactive PCR reactions for probe preparations were performed as described above with the addition of 2.5 μl of [α-32P]dCTP (3000 Ci/mmol). The G3PDH gene was amplified with Amplimer Sets (Clontech Laboratories, Inc., Palo Alto, CA). The PCR reaction was programmed as follows: initial denaturation at 94°C for 5 min, then 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, for 25 cycles; elongation at 72°C for 5 min; and refrigeration at 4°C. Northern blots were performed with ExpressHyb hybridization solution following the manufacturer’s instructions (Clontech Laboratories, Inc., Hercules, CA), and standard normalization procedures were performed to determine mRNAs relative expression.

Preparation of Primary Cell Culture and Infection by Retroviral Vectors. Primary keratinocytes were isolated from multiple human neonatal foreskins by standard technique (15). Keratinocytes were maintained in serum-free medium supplemented with human growth hormone and pituitary extract (Life Technologies, Inc.). The amphotropic packaging cell line (PA317) was used to produce recombinant retroviruses LXSX, HPV16 E6, HPV16 E7, or HPV16 E6/E7 under transcriptional control of the Moloney leukemia virus promoter-enhancer sequences (kindly provided by Dr. D. Galloway (Fred Hutchinson Cancer Research Center, Seattle, WA); Refs. 16 and 17). The LXSX vectors contain the gene conferring neomycin resistance directed from the SV40 promoter. Recombinant virus was generated according to a previously described procedure (15). Viruses produced from PA317 cells were used to infect passage 2 human neonatal foreskin keratinocytes. Infected cells were placed under G418 (200 μg/ml) selection for 48 h and then carried for 8 additional days until selection was complete. Expression of viral proteins and p53 was determined as described previously (15).

Statistical Analysis. The χ² test was used to evaluate the results, and a P < 0.05 was judged to be significant.

RESULTS

Identification of a Human mRNA Encoding an OXYB. To identify tumor markers that circulate in the blood, we first tested whether DD could detect a few tumor cells in blood. A 200-bp cDNA fragment specific to HeLa cells displayed differential expression in a 3-ml blood sample to which 100 HeLa cells were added. This cDNA
fragment was not detected in the control blood (Fig. 1A, d1). The cDNA was isolated and directly sequenced. It displayed 96% nucleotide sequence homology to a human OXYB-homologue gene isolated from male blood DNA and localized at chromosome 22q12.1-qter as reported in GenBank (PAC library RPCI-3, clone DJ430N08). We designate this OXYB-homologue gene as HLM for HeLa metastatic gene.

To confirm the DD data indicating differential HLM expression, Northern blot analysis was conducted on the HLM gene. Specific primers were designed to generate a fragment of 400 bp by RT-PCR, which was used as a probe in Northern blots. Fig. 1B shows the presence of a high-molecular-weight transcript (>5 Kb) of HLM gene up-regulated in HeLa cells as well as in blood samples containing \( 1 \times 10^3 - 10^4 \) HeLa cells. The gene was low or not detected in blood samples from three different healthy donors (Fig. 1B, Lanes d1-d3). This result is in accordance with the DD data (Fig. 1A).

Fig. 1C shows the alignment of the deduced HLM protein sequence with the human OXYB. The OXYB family members contain a moderately conserved domain of about 250 amino acid residues located in the COOH-terminal half of the mammalian OXYB and the yeast OSH1. A conserved peptide sequence found in this domain is considered to be a consensus motif. HLM contains high homology to the family members throughout the protein; it is 64% homologous to human OXYB (Fig. 1C), 63% homologous to OXYB rabbit, and 30% homologous to OSH1 yeast (data not shown).

**HLM RNA Expression Is Induced by HPV16 Oncoproteins E6 and E7.** Expression of HLM may be tied to the presence of the HPV oncogene. HeLa cells contain the HPV18 E6 and E7 oncoproteins. E6 is an oncoprotein that binds to the p53 protein and promotes its proteolysis by the ubiquitin pathway. p53 is known to block G1-S-phase cell cycle progression after DNA damage. E7 is a \( M_r \) 21,000 nuclear phosphoprotein that binds to the Rb protein and promotes cellular proliferation though the release of E2F. The abrogation of p53 functions by HPV proteins may contribute to cellular immortalization and transformation (18). E6 and E7 are the only HPV proteins required to immortalize primary human epithelial cells *in vitro* (19). We examined whether the expression of E6 and E7 in HFKs affected HLM mRNA levels. E7 expression as well as functional E6 production, determined by the analysis of p53 protein levels, was demonstrated by Western analysis in lysates from HFKs infected with viruses expressing HPV16 E6 and E7 (data not shown). Fig. 2 shows by semiquantitative RT-PCR a more than 10-fold increase in expression of HLM mRNA in HFK-expressing HPV16 E6 and E7 proteins in comparison with HFK infected with the retrovirus vector alone. The same results were obtained in three experiments using independent isolated HFK.

**The Increased Expression of the HLM Gene May Be a Useful Marker for Tumor Progression.** To identify whether expression of HLM is specific for HeLa cells and/or HPV-infected cells, we analyzed additional cervical cancer cell lines including C33A, CaSkI, and SiHa; a prostate cancer cell line, LNCaP; and a lung cancer cell line, NCI-H23, for the detection of HLM mRNA by RT-PCR. Two im-
mortalized but not transformed epithelial cell lines, 1321 and HaCat, were also analyzed. The HLM transcript was detected in all of the lines tested but at different levels (Fig. 3A). It was strongly up-regulated in four of six cancer cell lines including HeLa, C33A, SiHa, and LNCap and was moderately expressed in CaSki and NCI-H23 as well as in HaCat and 1321. HLM was not detectable in the HFK tested by RT-PCR in this experiment (Fig. 3A). HLM RNA detection was not restricted to the HPV-positive lines (1321, HeLa, CaSki, and SiHa), which suggests that pathways not dependent on HPV E6 or E7 can also lead to HLM expression, or else that these oncoproteins may act on common pathways responsible for the activation of the HLM gene in cancer.

We next conducted Northern analyses on total RNA from a normal cell line and several tumor-derived cell lines for HLM expression (Fig. 3B). HeLa was used as a positive control (Fig. 3C, Lane 10). HLM mRNA was present in all of the metastatic breast cancer cell lines (Fig. 3, B and C, Lanes 4, 5, 6, 7, and 9 respectively; Ref. 20). It was low in the nonmetastatic breast cancer cell lines and not detected in a normal human mammary epithelial cell line, 70N (Ref. 21; Fig. 3, B and C, Lanes 2, 3, 8, and 1 respectively). HLM RNA was also detected in SW620, a highly metastatic colon cancer cell line, but not in SW480, a nonmetastatic colon cancer cell line (data not shown). HLM was also up-regulated in a bladder cancer cell line, T24, (Fig. 3, B and 3C, Lane 11). These results show that HLM overexpression is not HeLa-specific and that there is a frequent association with lines derived from metastases.

The relative expression of HLM mRNA in 50 human normal tissues was analyzed using the Human RNA Master blot (Clontech Laboratories, Inc., Palo Alto CA). It is important to note that each sample is representative of 20 individuals who died of trauma. Fig. 4 shows that the HLM mRNA is expressed strongly in normal kidney, liver, and lung and was completely absent in normal uterus, mammary gland, peripheral blood, and lymph nodes. Its presence in human cancer cell lines and its absence or low expression in blood and most normal tissues suggest that HLM may be used as a molecular marker for some cancers.

To test HLM expression in human breast cancer, Northern blot analysis was conducted on mRNA from breast tissue samples from cancer patients. Normalization was performed to 36B4 (22). The results were quantitated and plotted as relative expression (Fig. 5). Whereas normal adjacent breast and normal breast luminal epithelial cell lines demonstrated no detectable HLM mRNA levels (Lanes N and L), 20% of the localized disease (Lanes 1–10) and 81% of the metastatic disease (Lanes 11–21) patients contained higher levels of HLM mRNA. Thus, HLM expression is significantly associated with metastatic disease (P = 0.03; Table 1). The seven metastatic lesions analyzed came from lymph nodes (four samples), hip (one sample), lung (one sample), or pleural wall (one sample). Although no information regarding the expression of HLM in normal hip or pleura was available, the Human RNA Master blot allowed us to examine its expression in normal lymph nodes and lung. 50% lymph node metastases expressed HLM; however, it was not observed in normal lymph nodes. This suggests that the HML up-regulation in these metastatic lesions came from tumor cells. Normal lung does express the gene at a high level. Thus, we could not confirm in lung that HLM expression in the lung metastasis is not due to the contamination of the sampled metastasis by normal tissue.

The detection of HLM in 42% of primary sites from either localized disease or metastatic disease (Fig. 5, Lanes 1–14) indicates that it may appear before detection of metastasis and that it could have a role in
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HLM as a Molecular Marker for Cancer Cells Circulating in Peripheral Blood. We analyzed by RT-PCR the expression of HLM in circulating tumor cells in cancer patients' blood. A limiting dilution analysis to determine the sensitivity of this method for detection of HLM mRNA was performed. In Fig. 6 a 400-bp band corresponding to HLM was observed and confirmed by direct sequencing (data not shown), even when 10 HeLa cells were mixed with 3 ml of blood. HLM was low or not detectable in the donor blood sample (Fig. 6, Lane d1). Thus, the limit of detection of HLM is approximately 1 cell in a million WBCs. We next tested blood samples from a total of 13 cancer patients collected before any antitumor treatment or surgery and from 9 tumor-free donors (Table 2). HLM was up-regulated in six of these samples. Information about these patients is described in Table 2. HLM was overexpressed in two of four blood samples from lung cancer patients with clinically localized disease. The data support the Northern blot results, in which we found expression of HLM in breast cancer patients with localized disease (Fig. 5). Four of eight metastatic blood samples were positive for HLM. It is also shown that in accordance with the cell line data, HLM is not tumor-specific. We found a highly significant association for the detection of HLM in lung cancer patients’ blood compared with tumor-free donors’ blood (P = 0.006). Although HLM mRNA expression in blood samples can be correlated with cancer, we could not find a significant association between HLM mRNA expression and tumor stage. We suggest that HLM can be a potential marker for tumor dissemination in peripheral blood.

Table 1  HLM mRNA-positive tumors

<table>
<thead>
<tr>
<th>Primary site</th>
<th>Stage</th>
<th>Histology</th>
<th>HLM mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Localized tumor</td>
<td>2/10</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Metastatic tumor</td>
<td>9/11</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Primary site</td>
<td>4/4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metastatic site</td>
<td>5/7</td>
<td>0.49</td>
<td></td>
</tr>
</tbody>
</table>

* α, number of tumors with high HLM mRNA levels; n, total number of patients analyzed.

Table 2  HLM levels in donors and patients’ blood samples

<table>
<thead>
<tr>
<th>No.</th>
<th>Tumor primary site</th>
<th>Stage</th>
<th>Histology</th>
<th>HLM RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lung</td>
<td>Localized</td>
<td>Small cell</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Lung</td>
<td>Localized</td>
<td>Adenocarcinoma</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Lung</td>
<td>Localized</td>
<td>Small cell</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Lung</td>
<td>Localized</td>
<td>Adenocarcinoma</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Lung</td>
<td>Metastatic</td>
<td>Adenocarcinoma</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Lung</td>
<td>Metastatic</td>
<td>Small cell</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Lung</td>
<td>Metastatic</td>
<td>Squamous</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>Lung</td>
<td>Metastatic</td>
<td>Small cell</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>Lung</td>
<td>Metastatic</td>
<td>Squamous</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>Lung</td>
<td>Metastatic</td>
<td>INA</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>Colon</td>
<td>Metastatic</td>
<td>Relapse</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>Breast</td>
<td>Metastatic</td>
<td>Adenocarcinoma</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>Breast</td>
<td>Metastatic</td>
<td>Relapse</td>
<td>-</td>
</tr>
</tbody>
</table>

α, detectable gene product; −, not detectable gene product; INA, information not available.

DISCUSSION

Our objectives were: (a) to detect a limited number of tumor cells in blood samples by DD. This method can be used as a tool to study cancer patients’ blood; (b) to find a marker for metastatic tumors that is initially a candidate for a blood assay for the early detection of metastasis. We describe the HLM gene, isolated from HeLa cells, that is overexpressed in breast and cervix cancer cell lines as well as in breast cancer tissue and lung cancer patients’ blood samples. In contrast, HLM mRNA expression in most normal human tissues or blood samples from tumor-free donors is low or not detectable either by Northern blot or by RT-PCR. We found the detection limit of one HeLa cell in 1 × 10⁶ WBCs. The detection of the HLM gene in blood from lung cancer patients was highly significant (P = 0.006). We observed HLM mRNA in three of four blood samples from patients with small cell lung cancer, which has a very aggressive clinical course with frequent widespread metastases and overall poor prognosis (23). The lack of detection of this marker in peripheral blood from control populations strongly suggests that the RT-PCR assay detects circulating tumor cells, although verification will be needed. The direct detection of neoplastic cells in the peripheral circulation was not formally addressed, and the effect of circulating tumor cells on gene expression of WBCs was also not evaluated.

Relative expression in breast tissue from patients with localized and metastatic disease demonstrated a significant association between HLM expression and metastasis (P = 0.03). In breast cancer metastases we found that the relative expression of HLM is similar in the primary site and metastatic site (P = 0.49). We also detected HLM in 2 of 10 breast cancer patients with localized disease. Additional experiments will determine whether the presence of HLM in primary sites is useful as a metastasis prediction factor.

The implication of HLM in the cell cycle has not been described for the previously described mammalian homologues, which play a role in sterol transport from lysosomes to the nucleus (8). The expression of HPV16 E6 and E7 proteins induced HLM mRNA expression in primary HFK. This result indicates that HLM expression is an early event in cell immortalization by high-risk HPV oncoproteins expres-
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REFERENCES


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