Immunohistochemical Variation of Human Equilibrative Nucleoside Transporter 1 Protein in Primary Breast Cancers

John R. Mackey, Lori L. Jennings, Marilyn L. Clarke, Cheryl L. Santos, Laith Dabbagh, Michaela Vsianska, Sheryl L. Koski, Robert W. Coupland, Stephen A. Baldwin, James D. Young, and Carol E. Cass

Departments of Experimental Oncology [L. L. J., M. L. C., C. L. S.], Medicine [J. R. M., S. L. K.], and Pathology [L. B., R. W. C.], Cross Cancer Institute, Edmonton, Alberta, T6G 1Z2 Canada; Masaryk Memorial Cancer Institute, 656 53 Brno, Czech Republic [M. V.]; School of Biochemistry and Molecular Biology, University of Leeds, Leeds, LS2 9JT United Kingdom [S. A. B.]; Departments of Physiology [J. D. Y], Biochemistry [C. E. C.], and Oncology [J. R. M., S. L. K., C. E. C.], University of Alberta, Edmonton, Alberta, T6G 2R7 Canada

ABSTRACT

Gemcitabine and capecitabine are nucleoside analogues used in chemotherapy strategies for the treatment of breast cancer. We previously demonstrated that deficiency in hENT1, the most abundant and widely distributed plasma membrane nucleoside transporter in human cells, confers high-level resistance to gemcitabine toxicity in vitro, whereas the relationship between hENT1 activity and capecitabine toxicity is unknown. To determine the relationship between capecitabine cytotoxicity and hENT1 abundance, cultured MDA-MB-435s human mammary carcinoma cells were exposed to graded concentrations of the capecitabine metabolite, 5'-DFUR, and staining intensity was scored on a 0–4+ scale. hENT1 staining intensity varied markedly among breast samples (4 with score 0, 5 with score 1+, 7 with score 2+, 14 with score 3+, 3 with score 4+), suggesting that at least 9 of the tumors were hENT1 deficient. We conclude that because hENT1 deficiency has previously been associated with nucleoside drug resistance, immunohistochemical staining of hENT1 warrants further study as a predictive tool for guiding the appropriate use of gemcitabine and capecitabine in the treatment of breast cancer.

INTRODUCTION

Nucleoside analogue chemotherapy has assumed increasing importance in the therapy of breast cancer. Two nucleoside analogues, gemcitabine (2',2'-difluorodeoxycytidine; Gemzar) and capecitabine (5'-deoxy-5-N-[(pentoxy)carbonyl]-cytidine; Xeloda), are now routinely used in the palliation of advanced breast cancer. Gemcitabine is administered i.v. and undergoes intracellular phosphorylation to the active metabolites gemcitabine diphosphate and gemcitabine triphosphate, leading to inhibition of ribonucleotide reductase and incorporation of gemcitabine triphosphate into DNA and RNA. Gemcitabine has activity in breast cancer, particularly when used in first-line therapy of metastatic disease (1, 2), with toxicities including fatigue, myalgias, fluid retention, and myelosuppression. Capecitabine is a p.o.-administered agent that has substantial activity in patients with locally advanced or metastatic breast cancer after failure of taxane or anthracycline chemotherapy (3), with toxicities including mucositis, diarrhea, and palmar-plantar erythrodysesthesia. Capecitabine is rapidly and extensively absorbed, metabolized in the liver by carboxylesterase to 5'-deoxy-5-fluorocytidine, and then converted to 5'-DFUR1 by cytidine deaminase in both the liver and tumor tissue. Finally, 5'-DFUR is converted to the cytotoxic 5-FU by thymidine phosphorylase in tumor tissue (4).

Because the biochemical targets of gemcitabine and capecitabine are intracellular, the obligatory first target in the production of cytotoxicity is permeation across the plasma membrane (5). Gemcitabine and physiological nucleosides are hydrophilic compounds and do not readily cross the plasma membrane by diffusion (6, 7). Efficient cellular uptake therefore requires the presence of specialized plasma membrane NT proteins (6). The recent isolation and functional expression of cDNAs encoding human (h) NT proteins (8–13) has identified two structurally unrelated protein families that are designated ENT and CNT, depending on whether they mediate, respectively, equilibrative (E) or concentrative (C) NT processes. The hENT proteins have

1 The abbreviations used are: 5'-DFUR, 5'-deoxy-5-fluorouridine; 5-FU, 5-fluorouracil; NT, nucleoside transport; NBMPR, nitrobenzylmercaptopurine ribonucleoside; mAb, monoclonal antibody.

Received 1/28/00; revised 7/16/01; accepted 7/27/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by the Alberta Breast Cancer Foundation, the Alberta Cancer Board Research Initiatives Program, the National Cancer Institute of Canada, and Roche Pharmaceuticals.

2 To whom requests for reprints should be addressed, at Department of Medicine, Cross Cancer Institute, 11560 University Avenue, Edmonton, Alberta, T6G 1Z2 Canada. Phone: (780) 432-8221; Fax: (780) 432-8888; E-mail: johnmack@cancerboard.ab.ca.
Fig. 1 Chemosensitivity of MDA-MB-435s breast cancer cells to 5-FU and 5'-DFUR. MDA-MB-435s cells were exposed to graded concentrations (0.1–200 μM) of 5-FU and 5'-DFUR in the presence or absence of the hENT1 transport inhibitor, NBMPR (100 nM) for 72 h. The IC₅₀ was determined with a CellTiter 96 proliferation assay and the concentration-effect curve generated using GraphPad Prism software. The data represent the mean of three separate experiments (bars, ± SD); bars are not shown where values were small and obscured by data points. A, □, 5-FU; A, 5-FU + NBMPR. B, △, 5'-DFUR; △, 5'-DFUR + NBMPR.

Table 1 Immunohistochemistry of cultured breast cancer cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Score</th>
<th>Binding sites</th>
<th>Gemcitabine uptake rate</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocytes</td>
<td>1+</td>
<td>ND</td>
<td>11,000 ± 0.7</td>
<td>(30, 44)</td>
</tr>
<tr>
<td>MCF-7</td>
<td>2+</td>
<td>35</td>
<td>113,500 ± 470</td>
<td>(30)</td>
</tr>
<tr>
<td>MDA-MB-435S</td>
<td>3+</td>
<td>95</td>
<td>182,500 ± 550</td>
<td>(30)</td>
</tr>
</tbody>
</table>

* Number of positively stained cells of 200 counted, expressed as a percentage.
* Number of NBMPR binding sites.
* Initial uptake rate of 10 μM radiolabeled gemcitabine, expressed as pmol/10⁶ cells/s, using methods described in (16).
* ND, not determined because erythrocyte membranes were used, not whole cells.

Materials and Methods

Materials. Synthetic hENT1 peptide conjugated to keyhole limpet hemocyanin was obtained from the Alberta Peptide Institute (University of Alberta, Alberta, Canada). Goat antimumouse antibody, horseradish peroxidase-labeled dextran polymer (DAKO EnVision+) was from DAKO Corporation (Carpinteria, CA). Capcitabine and 5'-DFUR were gifts from Roche Pharmaceuticals (Basel, Switzerland). 5-FU was from Pharmacia & UpJohn, Inc. The murine myeloma cell line (PC/NSI/1-AG4-1) and the human MCF-7 and MDA-MB-435s breast cancer cell lines were from the American Type Culture Collection (Rockville, MD). Tissue culture growth medium and supplements were from Life Technologies, Inc. (Burlington, Ontario, Canada) and the CellTiter 96 proliferation assay kit was from Promega Corporation (Madison, WI). All other reagents used were analytical grade and commercially available.

Antibody Production. mAbs specific for the hENT1 protein were produced by immunization of mice with a synthetic peptide-keyhole limpet hemocyanin conjugate. The synthetic peptide corresponded to amino acids 254–271 of the predicted intracellular loop between transmembrane domains 6 and 7 of hENT1. The production and validation of the antibodies were based on methods adapted from Harlow and Lane (25) and are described elsewhere (26). Briefly, hybridomas were produced by fusion of splenocytes with the murine myeloma nonsecreting cell line, PC/NSI/1-AG4-1, and maintained in growth medium that consisted of RPMI 1640 supplemented with 20% heat-inactivated fetal bovine serum. Hybridomas were selected by their ability to grow in medium that contained 100 μM hypox-
Immunohistochemistry for hENT1

Embedding material, snap-frozen in a dry ice-methanol bath, and were harvested by trypsinization, pelleted, resuspended in OCT to simulate a biopsy specimen. Exponentially growing cells modified Richardson-Bloom (27) overall histological grade was IV in 3 patients. Tumor size ranged from 0.2 to 7.0 cm. Twenty-three patients, stage II in 18 patients, stage III in 2 patients, and stage IV in 2 patients. Tumor size ranged from 0.2 to 7.0 cm. Twenty-four patients had axillary lymph node-negative disease. The modified Richardson-Bloom (27) overall histological grade was grade 1 in 6 cases, grade 2 in 10 cases, and grade 3 in 17 cases. Ten cases lacked detectable estrogen receptors, and 13 cases lacked detectable progesterone receptors by immunohistochemistry. Nine cases had peritumoral lymphovascular invasion. Tissue specimens were suspended in OCT and snap-frozen in a dry ice-methanol bath. Cryostat sections (4–6 μm thick) were picked up on glass microscope slides and dried at room temperature overnight, followed by a 10-min fixation in acetone, and then air-dried for 5 min. Tissue sections were incubated with appropriate dilutions of anti-hENT1 mAbs (10 μg/ml) in a room temperature humidity chamber for 30 min. The sections were then rinsed in PBS, immersed in buffer for 5 min, incubated with goat antimouse dextran conjugate for 30 min followed by diaminobenzidine solution, rinsed, counterstained with hematoxylin, dehydrated through graded alcohols and xylene, and coverslipped. Negative controls were provided by omitting the primary antibody and by peptide adsorption of the primary antibody.

Immunostaining was assessed and scored subjectively by a single pathologist (R.C.) blinded to clinical characteristics and outcomes. hENT1 staining was scored on a 0–4+ scale based on the relative intensities of staining observed in benign breast duct/lobules and capillary endothelial cells present within biopsy tissues. These internal reference points were then used as internal controls between slides and samples and for the staining procedure. Invasive and in situ carcinoma were evaluated by comparison with the internal controls. The cytological pattern and the percentage of stained cells were also evaluated and recorded where applicable.

Statistical Analysis. hENT1 staining intensities were studied for correlations with pathological features (nuclear, architectural, mitotic or overall histological grade, vascular invasion, tumor size, hormone receptor status), clinical characteristics (tumor stage, patient age), and age of the tissue specimen. Spearman correlation coefficients were calculated with SAS Proprietary Software (Release 6.12; SAS Institute Inc., Cary, NC). The level of significance was set at 5%, using two-sided analysis.

RESULTS

Cytotoxicity of the Capecitabine Metabolites 5’-DFUR and 5-FU. To examine the role of the hENT1 NT in the uptake of 5’-DFUR into tumor cells in vitro, MDA-MB-435s cells were exposed to graded concentrations of 5’-DFUR or 5-FU for 72 h in the absence or presence of the NT inhibitor.
NBMPR at a concentration of 100 nM, which is known to inhibit the activity of the hENT1-transporter (Fig. 1). As expected, NBMPR provided little protection against the cytotoxic effects of 5-FU (1.3-fold) because this compound, a nucleobase, is not a substrate of the hENT1 transporter (Fig. 1A). IC_{50}'s for 5-FU in the absence or presence of NBMPR were 3.0 ± 0.3 and 3.8 ± 0.1 µM, respectively. However, significant protection was demonstrated against 5'-DFUR in the presence of NBMPR, suggesting that hENT1-mediated uptake enhanced its cytotoxic effect (Fig. 1B). The IC_{50} for 5'-DFUR in the absence of NBMPR was 18.2 ± 3.2 µM.

Table 2  Immunohistochemistry of breast tumor tissue

<table>
<thead>
<tr>
<th>Number of patient samples</th>
<th>Score&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>(total n = 33)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>1+</td>
</tr>
<tr>
<td>7</td>
<td>2+</td>
</tr>
<tr>
<td>14</td>
<td>3+</td>
</tr>
<tr>
<td>3</td>
<td>4+</td>
</tr>
</tbody>
</table>

<sup>a</sup> Scoring was based on the range of intensities observed for hENT1 staining of the negative (0) and strongly positive (4+) internal controls.

Development of the Immunostaining Technique.

Formalin-fixed samples of pelleted cultured breast cancer cells and formalin-fixed, paraffin-embedded breast tissue gave markedly inconsistent staining despite the use of antigen-retrieval techniques involving high-temperature treatment of tissues with aqueous salt solutions (28, 29). Therefore, this immunocytochimical method did not allow meaningful analysis of formalin-fixed, paraffin-embedded tumor blocks. However, uniform and reproducible staining was achieved with frozen cell pellets and frozen tissues treated with anti-hENT1 mAb (10 µg/ml) and stained as described in “Methods and Materials.”

Erythrocyte membranes and the cultured breast cancer cell lines MCF-7 and MDA-MB-435s were stained and scored using the anti-hENT1 mAbs (Table 1). The increased staining intensities and percentages of cells stained were correspondingly higher in cells with greater numbers of hENT1 molecules and with correspondingly higher rates of gemcitabine uptake. The number of plasma membrane hENT1 proteins for each of these cell types is known from previous experiments and is based on the measurement of the number of NBMPR-binding sites with a membrane-impermeant probe (30). The ability to detect hENT1 proteins in erythrocyte membranes suggested that the immunostaining method was very sensitive, with a lower limit of detection ≤11,000 hENT1 molecules per cell membrane.

Immunohistochemistry of Breast Tumor Tissue.

There was no apparent correlation with age of the pathological specimen (range, 1–9 years) and hENT1 staining intensity, suggesting that antigen detection was not impaired by prolonged freezing at −70°C. hENT1 immunohistochemical staining intensity varied markedly among breast samples (Table 2). All control slides, prepared either with unrelated isotypic antibodies or without the anti-hENT1 mAbs, revealed no background immunoperoxidase staining and therefore served as appropriate negative controls. Statistical analysis showed that hENT1 staining did not correlate with the pathological features of the invasive breast adenocarcinomas (nuclear, architectural, mitotic or overall histological grade; vascular invasion, tumor size, hormone receptor status) or clinical features (tumor stage, patient age; Table 3).

Staining was observed in normal breast ducts/lobules and in capillary endothelium (Fig. 2). Myoepithelial cells within the normal lobules exhibited the most intense staining, and this intensity was arbitrarily given a score of 4+. The less intensely staining endothelium was given a score of 2+. Definite staining of tumor cells with intermediate intensities was assigned as 3+, whereas weak but definite positive staining was given a score of 1+. Some duct/lobular epithelial cells also exhibited a 2+ intensity of staining, predominantly of the basal and lateral cell membranes.

In all cases the in situ and invasive carcinomas stained uniformly, with most cases having predominantly membrane staining (Fig. 3). The intensity of staining of MCF7 cells approximated that of endothelia (2+), whereas that of MDA-MB-435s cells (Fig. 4) was slightly more intense (3+) but less than duct/lobe cells.

**DISCUSSION**

The number of hENT1 NT proteins on human plasma membranes varies greatly among human cells and cell lines. Cellular hENT1 abundance can be estimated from the number of high-affinity binding sites for radiolabeled NBMPR and ranges from a low of 1000 binding sites per polymorphonuclear cell (31) to a high of >10^7 sites per BeWo choriocarcinoma cell (32). In cultured human cancer cells, cellular hENT1 protein levels may approximately double between the G<sub>1</sub> and G<sub>2</sub>-M phases of the cell cycle (33, 34), indicating that the cellular content of hENT1 is regulated in a coordinated way with the cell cycle. High cellular proliferation rates are associated with higher abundance of hENT1 protein (35, 36). Leukapheresis (37), granulocyte macrophage colony-stimulating factor stimulation (38), and cytotoxic nucleoside exposure (39) have been reported to cause 2–6-fold increases in hENT1 protein in human leukemia cells. There is some evidence that hENT1 abundance is increased in malignant solid tumors when compared with their tissues of origin for breast, stomach, and colorectal adenocarcinomas (40). However, this latter study was performed with NBMPR equilibrium binding assays of crude tumor plasma.

Table 3  Statistical analysis of hENT1 staining

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Correlation coefficient</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient age</td>
<td>0.06</td>
<td>0.73</td>
</tr>
<tr>
<td>Tumor stage</td>
<td>0.17</td>
<td>0.31</td>
</tr>
<tr>
<td>Tumor size</td>
<td>0.12</td>
<td>0.50</td>
</tr>
<tr>
<td>Vascular invasion</td>
<td>−0.01</td>
<td>0.93</td>
</tr>
<tr>
<td>Overall histological grade</td>
<td>0.08</td>
<td>0.64</td>
</tr>
<tr>
<td>Nuclear grade</td>
<td>0.08</td>
<td>0.67</td>
</tr>
<tr>
<td>Mitotic grade</td>
<td>0.16</td>
<td>0.41</td>
</tr>
<tr>
<td>Architectural grade</td>
<td>−0.23</td>
<td>0.23</td>
</tr>
<tr>
<td>Estrogen receptor</td>
<td>−0.06</td>
<td>0.73</td>
</tr>
<tr>
<td>Progesterone receptor</td>
<td>−0.15</td>
<td>0.38</td>
</tr>
</tbody>
</table>

Spearman correlation coefficients and P values for the hENT1 staining scores ranked against pathological or clinical features for all 33 patients.
membrane preparations, and the authors were unable to distinguish the contributions of malignant glandular cells from that of benign stromal cells, such as tumor-associated capillaries.

In the present study, hENT1 immunohistochemistry analysis of breast capillary endothelial cells revealed universal staining. This result was consistent with the previous identification of NBMPR-sensitive nucleoside transport and NBMPR-binding sites on cultured human umbilical vein endothelial cells (41) and with a prior immunolocalization study that identified hENT1 on the luminal surface of the human fetal-placental vasculature (42). Because the staining intensities of vascular endothelial cells were uniform, both in tumor-associated and nontumor-associated breast capillaries, endothelial cell staining provided an appropriate internal control for the interpretation of hENT1-stained tissues. Although breast carcinoma cells have previously been suggested to have higher cellular hENT1 abundance than surrounding benign epithelial cells (40), this conclusion warrants scrutiny. Because breast cancers commonly produce tumor-associated angiogenic substances, leading to increased microvessel densities (43), the increased number of NBMPR-binding sites observed in solid tumor-derived membranes, when compared with adjacent normal tissues, might simply be a byproduct of a higher proportion of endothelial cells.

Invasive adenocarcinoma cells from 33 primary breast tumors revealed marked intertumoral variability in hENT1 immunohistochemistry staining intensity. Immunohistochemical staining intensities were independent of other pathological and clinical features, including tumor grade, stage, and clinical outcomes. Because hENT1 deficiency confers high-level gemcitabine resistance in vitro (16), it is possible that hENT1-immunohistochemistry might identify those patients with gemcitabine-resistant disease; hENT1 immunohistochemistry therefore warrants investigation as a novel predictive marker for gemcitabine chemotherapy. hENT1 immunohistochemistry may also hold promise for the identification of capecitabine-resistant breast cancers. We found that a low concentration (100 nM) of NBMPR, the tight-binding inhibitor of hENT1 function, protected cultured MDA-MB-435s cells from cytotoxicity produced by 5’-DFUR, a metabolite of capecitabine. These results suggested that hENT1 deficiency may contribute to clinical capecitabine resistance.

We conclude that, because hENT1 deficiency has been associated with nucleoside drug resistance (16, 18–23), immu-

Fig. 3 Cryostat-frozen sections of invasive ductal carcinoma showing negative control (A) and positive immunostaining (B). The round nuclei (stained blue by hematoxylin) are surrounded by positively stained cytoplasm and cytoplasmic membranes. At lower magnification (C), a centrally located, diagonally oriented breast duct is expanded by intensely positive in situ carcinoma. Accompanying invasive ductal carcinoma is observed in the lower right-hand corner and, to a lesser extent, in the upper left-hand corner, is also strongly stained positive for hENT1.

Fig. 4 Cryostat-frozen sections of MDA-MB-435 breast carcinoma cell line pellets. A, negative control; B, positive staining for hENT1.
Acknowledgments

We thank Roche Pharmaceuticals for providing the capcetabin and 5'-DFUR. Miguel Cabrita for preparation of the erythrocyte membranes, Linda Harris and Mary Shirton for assistance with manuscript preparation, and John Hanson for statistical analysis.

References

Immunohistochemical Variation of Human Equilibrative Nucleoside Transporter 1 Protein in Primary Breast Cancers

John R. Mackey, Lori L. Jennings, Marilyn L. Clarke, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/8/1/110

Cited articles
This article cites 37 articles, 13 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/8/1/110.full.html#ref-list-1

Citing articles
This article has been cited by 13 HighWire-hosted articles. Access the articles at:
/content/8/1/110.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.