The Absence of Human Equilibrative Nucleoside Transporter 1 Is Associated with Reduced Survival in Patients With Gemcitabine-Treated Pancreas Adenocarcinoma

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ABSTRACT

Purpose: Gemcitabine monotherapy is the standard palliative chemotherapy for pancreatic adenocarcinoma. Gemcitabine requires plasma membrane nucleoside transporter proteins to efficiently enter cells and exert it cytoxicity. *In vitro* studies have demonstrated that deficiency of human equilibrative nucleoside transporter 1 (hENT1), the most widely abundant and distributed nucleoside transporter in human cells, confers resistance to gemcitabine toxicity, but the distribution and abundance of nucleoside transporters in normal and malignant pancreatic tissue is unknown.

Experimental Design: We studied tumor blocks from normal pancreas and 21 Alberta patients with gemcitabine-treated pancreatic cancer. Immunohistochemistry on the formalin-fixed, paraffin-embedded tissues was performed with specific hENT1 and human Concentrative Nucleoside Transporter 3 monoclonal antibodies and scored by a pathologist blinded to clinical outcomes.

Results: hENT1 was detected in normal Langerhan cells and lymphocytes but not in normal glandular elements. Patients in whom all adenocarcinoma cells had detectable hENT1 had significantly longer median survivals from gemcitabine initiation than those for whom hENT1 was absent in a proportion (10 to 100%) of adenocarcinoma cells (median survival, 13 *versus* 4 months, $P = 0.01$). Immunohistochemistry for human Concentrative Nucleoside Transporter 3 revealed moderate to high-intensity staining in all adenocarcinoma tissue samples.

Conclusions: Patients with pancreatic adenocarcinoma with uniformly detectable hENT1 immunostaining have a significantly longer survival after gemcitabine chemotherapy than tumors without detectable hENT1. Immunohistochemistry for hENT1 shows promise as a molecular predictive assay to appropriately select patients for palliative gemcitabine chemotherapy but requires formal validation in prospective, randomized trials.

INTRODUCTION

Cytotoxic nucleoside analogues were among the first chemotherapeutic agents introduced for the medical treatment of cancer. This family of antimetabolites includes pyrimidine nucleoside derivatives that have been used for many years for treatment of hematologic malignancies. However, among the pyrimidine analogues, 2′,2′-difluorodeoxycytidine (gemcitabine) has considerable activity against solid tumors, including pancreatic, lung, breast, and bladder cancer (1–3).

The cytotoxic effects of gemcitabine are multifold. Gemcitabine is phosphorylated by deoxycytidine kinase to its mononucleotide in a rate-limiting step. Subsequent nucleotide kinases convert gemcitabine monophosphate to its active metabolites, gemcitabine diphosphate and gemcitabine triphosphate. Through masked DNA chain termination, gemcitabine triphosphate incorporates into DNA, with a subsequent addition of a natural nucleotide, thereby making the strand less vulnerable to DNA repair by base pair excision (4, 5). Gemcitabine also has self-potentiating mechanisms that achieve higher intracellular concentrations and increase cytotoxicity. The *de novo* DNA synthesis pathway is blocked through inhibition of ribonucleotide reductase by gemcitabine diphosphate (6). At high cellular concentrations, gemcitabine triphosphate inhibits deoxycytidine monophosphate deaminase and cytidine triphosphate synthethase, thereby lowering the opposing deoxycytidine triphosphate pool (7, 8).

Because the pharmacological targets of nucleoside analogues are intracellular, the obligatory first step for manifestation of cytotoxicity is permeation of the analogues through the plasma membrane (9). Gemcitabine and physiologic nucleosides are hydrophilic, and diffusion through the plasma membrane lipid bilayer is slow. Efficient cellular uptake therefore requires the presence of specialized integral membrane nucleoside transporter proteins (10, 11). Two general processes of nucleoside transport have been identified: the equilibrative bi-directional facilitators and the concentrative sodium/nucleoside symporters (9). The equilibrative nucleoside transporter (ENT) proteins differ in their sensitivities to inhibition from nanomolar concen-

trations of the nucleoside transport inhibitor nitrobenzylmercaptopurine ribonucleoside. Human ENT1 (hENT1) is sensitive to nanomolar concentrations of nitrobenzylmercaptopurine ribonucleoside, whereas hENT2 is not readily inhibited by nitrobenzylmercaptopurine ribonucleoside. The ENT proteins are found in most cell types. Molecular cloning of cDNA encoding transporter proteins has identified three sodium-dependent concentrative nucleoside transporter (CNT) proteins. Human CNT1 (hCNT1) transports pyrimidine nucleosides more favorably, whereas hCNT2 transport purine nucleosides and uridine. The recently cloned hCNT3 is a broad-specificity nucleoside transporter that accepts both pyrimidine and purine nucleosides as permeants (12, 13). Of the three hCNT proteins, hCNT3 RNA expression appears to have the widest tissue distribution; hCNT3 transcripts are found in the pancreas, bone marrow, mammary gland, trachea, liver, and prostate and regions of the intestine, brain, and heart (12).

Kinetic studies of human cell lines with defined nucleoside transporter processes have demonstrated that gemcitabine intracellular uptake is mediated by hENT1, hENT2, hCNT1, and hCNT3 (11, 13–15). However, the major routes for transporting gemcitabine are hENT1 and, to a lesser extent, hCNT1 and hCNT3 (11, 13–15). Previously, it has been demonstrated that ENT1-deficient cells are resistant to cytotoxic nucleosides *in vitro* (14, 16–21). With the advent of molecular and immunologic probes for nucleoside transporter proteins, the differential expression of nucleoside transporters in normal and tumor tissue can now be studied (22–24).

It is anticipated that 30,700 new cases of pancreatic adenocarcinoma will be diagnosed within the United States in 2003 (25). The majority of these patients have unresectable disease or will recur after surgery. For these patients, gemcitabine monotherapy remains the standard palliative first-line chemotherapy regimen with toxicities, including myelosuppression, fatigue, myalgias, and fluid retention (3). Unfortunately, the majority of patients with gemcitabine-treated pancreatic adenocarcinoma fail to derive benefit from chemotherapy. Because no clinical or molecular marker has been shown to predict benefit from gemcitabine therapy, patients are treated empirically until evidence of disease progression of worsening performance status.

We hypothesized that patients with nucleoside transporter-deficient pancreatic adenocarcinoma would derive minimal benefit from palliative gemcitabine. Therefore, we performed immunohistochemical staining for hENT1 and hCNT3 protein abundance in gemcitabine-treated pancreatic adenocarcinomas and related protein abundance to overall survival from initiation of chemotherapy.

MATERIALS AND METHODS

Patients. Inclusion in this study required each of the following criteria: (*a*) histologic diagnosis of pancreatic adenocarcinoma; (*b*) tumor samples derived from core biopsy or surgical resection specimens suitable for immunohistochemical assessment; (*c*) no gemcitabine or radiotherapy before the tissue sampling; and (*d*) treatment with gemcitabine at an Alberta Cancer Board facility between September 1998 and December 2002. Patients were identified by a computer search of the provincial pharmacy database, and medical records of each

candidate were searched for eligibility. Approval to conduct the study was granted by the institution's research ethics board. Fifty-nine candidates were identified by the computerized search of the pharmacy database, and 21 patients met all eligibility criteria. The majority of patients excluded did not receive gemcitabine as first-line chemotherapy or received it with other chemotherapeutic agents.

Chemicals and Reagents. Anti-hENT1 and anti-hCNT3 monoclonal antibodies were developed and characterized as described previously (23, 24). Goat antimouse antibodies and horseradish peroxidase-labeled dextran polymer (DAKO EnVision+) were purchased from DAKO Corporation (Carpinteria, CA). All other reagents were of analytical grade and commercially available.

Tissue Preparation and Immunostaining. Twenty-one formalin-fixed, paraffin-embedded pancreatic tumor sections (4 to 6 μm) were deparaffinized with three immersions in xylene baths (10 minutes each) followed by serial washes in graded alcohol from 100 to 50%. After rinsing in water, slides were placed in 250 mL of high pH 1× DAKO target antigen retrieval solution and microwaved in TT-mega Milestone (ESBE Scientific, Markham, Ontario, Canada) under control temperature and high pressure for 10 minutes at 100°C. After cooling in water for 6 minutes, slides were rinsed with water and peroxidase blocked in 3% H_2O_2 solution with methanol for 10 minutes then washed in running water for 10 minutes. PBS (pH 7.2) was used for rinsing before incubation with appropriate dilutions of anti-hENT1 or anti-hCNT3 monoclonal antibodies. Slides with anti-hENT1 were incubated in a humidified chamber overnight at 4°C, whereas slides with anti-hCNT3 sections were incubated for 30 minutes at room temperature. The sections were then rinsed with PBS, immersed in buffer for 5 minutes, incubated with goat antimouse dextran conjugate (DAKO Envision+) for 30 minutes, followed by soaking in PBS. DAKO diaminobenzidine liquid chromagen was placed on the samples for 5 minutes and rinsed, after which, the slides were soaked in 1% $CuSO_4$ for another 5 minutes. Subsequently, the sections were rinsed, counterstained with hematoxylin, dehydrated through graded alcohol and xylene, and finally coverslipped. Negative controls were provided by omitting the primary antibodies.

A single pathologist (R. Lai), blinded to clinical characteristics and outcomes, assessed and scored the hENT1 and hCNT3 immunostaining intensities on a 0 to 2+ scale. Scoring for hENT1 was based on relative intensities of staining of the pancreatic tumor with reference to the normally strong hENT1 staining of cell membranes within the islets of Langerhan cells and lymphocytes. Scoring for hCNT3 was compared with the normally strong but variable staining intensities of exocrine parenchymal cells. These internal references were then used as internal positive controls between slides and samples as well as for the staining procedure. Pancreatic tumor tissue was then evaluated by comparison with the internal controls. The most intense hENT1 and hCNT3 staining was given a score of 2+, whereas the absence of staining was scored 0. Intermediate staining between 0 and 2+ was scored 1+. Samples with regions of varying staining intensities of hENT1 and hCNT3 were scored and the percentages of each staining intensity were recorded.

Statistical Analyses. Overall survival from the date of initiation of gemcitabine until the date of death from any cause were calculated according to the Kaplan-Meier method and compared by the log-rank test based on the pattern of hENT1 immunostaining. Univariate analysis for correlation between postchemotherapy survival and clinical characteristics included patient age, sex, stage of disease at gemcitabine initiation, and Eastern Cooperative Oncology Group performance status at the prechemotherapy assessment. Results were considered significant at $P \leq 0.05$.

RESULTS

Patient Population. We studied tumor blocks from 21 northern Alberta patients (11 male, 10 female) with gemcitabine-treated pancreatic adenocarcinoma (Table 1). The median age was 58.0 years [95% confidence interval (CI), 51–64]. Sixteen patients (76%) in this cohort presented with American Joint Committee on Cancer stage IV disease at the time of initial diagnosis. The median survival of the entire group from diagnosis of pancreatic cancer was 11.0 months (95% CI, 6.8–17.5). At the time of initiation of first-line chemotherapy with gemcitabine, stage IV disease was present in 20 patients. In the lone exception, gemcitabine was started for stage III disease. The median survival from initiation of gemcitabine chemotherapy was 5.0 months (95% CI, 2.8–12.2). Three patients were alive at the time of the analysis in August 2003.

hENT1 Immunohistochemistry of Pancreatic Tumor Tissue and Survival from Gemcitabine Initiation. Of the 21 tissue samples, 9 samples had uniformly detectable hENT1 immunostaining (intensity scores of 1+ and/or 2+) with no heterogeneous regions lacking hENT1 (Fig. 1). Twelve samples possessed a proportion (range 10 to 100%) of adenocarcinoma cells without detectable hENT1 (intensity score 0).

Patients with regions of tumor tissue lacking hENT1 immunostaining had a median survival of 4.0 months (95% CI, 1.5–6.9) from initiation of gemcitabine chemotherapy. In contrast, patients with uniformly detectable hENT1 staining had a 3-fold longer median survival of 13.0 months (95% CI, 4.2–20.4). Kaplan-Meier analysis of survival from gemcitabine initiation revealed a significant separation in the survival curves ($P = 0.01$; Fig. 2).

Analysis of Survival. Univariate analysis revealed no significant relationships between patient clinical characteristics and overall survival from diagnosis or between clinical characteristics and duration of survival after gemcitabine initiation. Parameters examined included patient age, sex, Eastern Cooperative Oncology Group performance status at diagnosis, and stage at gemcitabine initiation (Table 1). The single patient with stage American Joint Committee on Cancer stage III disease survived only 5 months from the initiation of gemcitabine, and her tumor lacked detectable hENT1 on immunostaining. Additionally, the median time between diagnosis of pancreatic cancer and the initiation of gemcitabine was well-balanced in both groups: 3.0 months (range 1, to 22 months) in those patients who tumors lacked detectable hENT1 and 2.5 months (range, 0.5 to 18 months) in those patients with uniformly staining hENT1 adenocarcinoma. In brief, apart from hENT1 staining, there were no other parameters identified that accounted for the significant separation in survival curves.

hCNT3 Immunohistochemistry of Pancreatic Tumor Tissue. Immunohistochemistry with anti-hCNT3 monoclonal antibodies demonstrated uniform staining in all adenocarcinoma cells (data not shown). Classification of tumors could not, therefore, be undertaken.

DISCUSSION

Gemcitabine is a pyrimidine nucleoside analogue that requires protein-mediated plasma membrane uptake to efficiently enter cells and access its intracellular targets. Studies in cultured human cells show that hENT1 is the major means by which gemcitabine enters cells and that hENT1 deficiency is associated with gemcitabine resistance (14). Similarly, hCNT3, a transporter with high levels of mRNA expression in normal pancreatic tissue, can mediate gemcitabine entry (12). However, in recent studies performed on four cultured human pancreatic cancer lines, hENT1 was found to be the major gemcitabine transporter, and although low levels of CNT-type gemcitabine transport were detected, sodium-dependent gemcitabine uptake disappeared when cells grew to confluence (15). With the recent development of nucleoside transporter-specific monoclonal antibodies, it is now possible to study the abundance and distribution of hENT1 and hCNT3 in formalin-fixed tissues (23, 26). We studied hENT1 and hCNT3 protein abundance in gemcitabine-treated pancreatic adenocarcinomas and sought relationships with clinical outcome.

Immunohistochemical identification of the hENT1 protein in pancreatic tumor tissue revealed two patterns of staining, either (*a*) uniformly positive staining of adenocarcinoma cells or (*b*) heterogeneous regions lacking hENT1 in a proportion of

Table 1 Clinical characteristics of study patients

No. of patients	21
Age (y)	
Median	58.0 (95% CI, 51.0–64.0)
Range	39.0–72.0
Males:Females	11:10
Eastern Cooperative Oncology Group performance status	
Median	1
Range	0–3
American Joint Committee on Cancer stage at diagnosis, *n* (%)	
I	1 (4.8)
II	2 (9.5)
III	2 (9.5)
IVA	7 (33.3)
IVB	9 (42.9)
Stage at gemcitabine start, *n* (%)	
III	1 (4.8)
IVA	9 (42.9)
IVB	11 (52.4)
Survival from initial diagnosis (mo)	
Median	11.0 (95% CI, 6.8–17.5)
Range	1.0–36.0
Survival from gemcitabine initiation (mo)	
Median	5 (95% CI, 2.8–12.2)
Range	0.5–25.0

Fig. 1 Immunohistochemical staining of normal pancreas and pancreatic adenocarcinoma for hENT1 protein. *A*, normal pancreas showing strong immunostaining in the normal islets of Langerhan (*arrows*) but not in the normal parenchyma. Omission of the primary antibody yielded no immunostaining (data not shown). *B*, pancreatic adenocarcinoma showing strong plasma membrane immunostaining for hENT1 (*arrows*), infiltrating normal pancreatic parenchyma with glandular elements negative for hENT1. *C*, pancreatic adenocarcinoma lacking hENT1 staining (*arrows*), whereas adjacent lymphocytes demonstrate staining and provide a positive internal control.
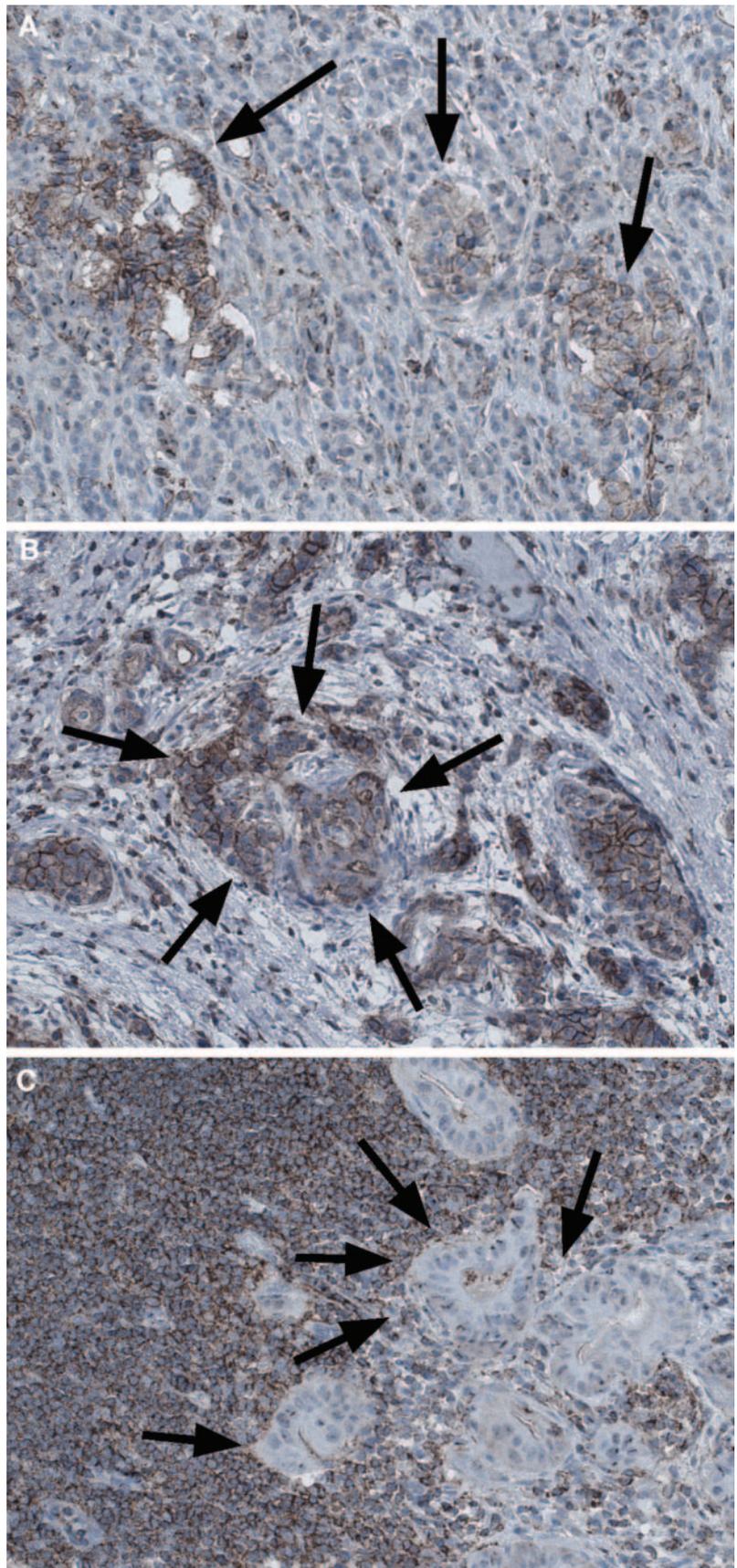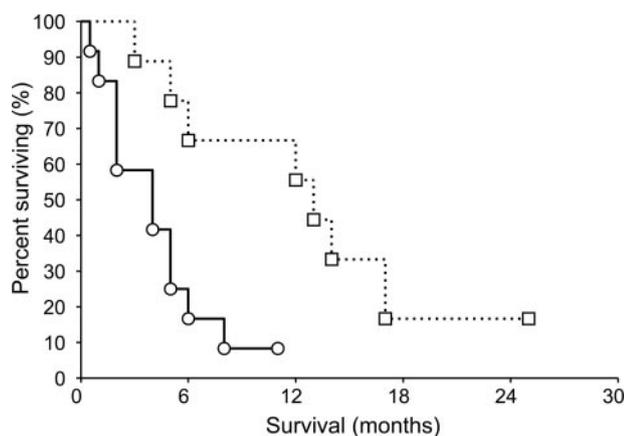
Fig. 2 Kaplan-Meier estimate of survival in gemcitabine-treated pancreatic cancer patients. Patients for whom all adenocarcinoma cells had detectable hENT1 (□ and *dashed line*) had significantly longer survival than those patients with heterogeneous areas of adenocarcinoma cells lacking hENT1 (○ and *continuous line*; median survival 13 *versus* 4 months; $P = 0.01$).

adenocarcinoma cells (range, 10 to 100%). Because the staining intensities of islet of Langerhan cells and lymphocytes were consistent, both in tumor-associated tissue and controls, islets and lymphocytes provided an appropriate internal positive control for the interpretation of hENT1-stained pancreatic tissue. The heterogeneity of hENT1 staining in some tumors suggests that subpopulations of pancreatic cancer cells may vary in their nucleoside transport capacities and sensitivities to gemcitabine. Low cellular proliferation rates have been linked to reduced abundance and activity of the hENT1 protein in leukemic cells and lymphocytes (27, 28). Similarly, low expression of hENT1 message in acute myelogenous leukemia was associated with shortened disease-free survival after cytarabine induction therapy (29, 30). Cells lacking functional hENT1 by mutagenesis (31) or pharmacological inhibition of hENT1 (9, 14, 24) are rendered nucleoside resistant.

This is the first immunohistochemical assessment of nucleoside transporters in a nucleoside chemotherapy-treated population. Our results suggest that immunohistochemical staining for hENT1 abundance within pancreatic tumor cells is a prognostic marker for survival after gemcitabine therapy. Patients with detectable hENT1 in all pancreatic adenocarcinoma cells had a statistically longer survival from initiation of gemcitabine monotherapy (median survival of 13.0 months) than patients whose adenocarcinoma cells lacked detectable hENT1 (median survival of 4.0 months; Fig. 2). Gemcitabine has a short serum half-life after the standard 30-minute bolus infusion (32), and our findings suggest that populations of cells with lower hENT1 protein abundance may be relatively gemcitabine resistant due to reduced intracellular accumulation. Conversely, increased hENT1 abundance and activity may facilitate nucleotide synthesis through nucleoside salvage pathways but may also facilitate efficient cellular entry of gemcitabine and confer increased cytotoxicity. To prove whether hENT1 is a predictive assay for benefit from gemcitabine therapy and not simply a marker of prognosis, prospective molecular correlative studies in trials where patients are randomized either to gemcitabine or a nonnucleoside therapy are required.

Immunohistochemistry using the specific hCNT3 monoclonal antibodies resulted in all adenocarcinoma cells being stained consistently. It is uncertain, therefore, whether the abundance of hCNT3 affects clinical outcome in patients with pancreatic adenocarcinoma because our assay lacked sufficient dynamic range to identify differences among samples. Because hCNT3 also accepts gemcitabine as a permeant and was detected in pancreatic adenocarcinoma cells (data not shown), it could be argued that its presence might offset any deficiencies in hENT1. However, because of the potentially different sensitivities of the hENT1 and hCNT3 immunohistochemical assays, our study does not allow comparison of the relative numbers of each of the two proteins in a given tumor.

We conclude that hENT1 immunostaining of pancreatic tumor cells serves as a prognostic marker for survival after gemcitabine treatment of pancreatic cancer. Uniformly detectable hENT1 immunostaining in pancreatic adenocarcinoma cells was associated with prolonged survival after treatment with gemcitabine, whereas tumors without detectable hENT1 had a short median survival. If confirmed in a larger prospective series, this assay may assist clinicians to select those patients most likely to experience the survival benefit of palliative gemcitabine monotherapy in pancreatic adenocarcinoma by relating it to pretreatment hENT1 protein abundance as assessed by a hENT1 immunohistochemical assay.

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