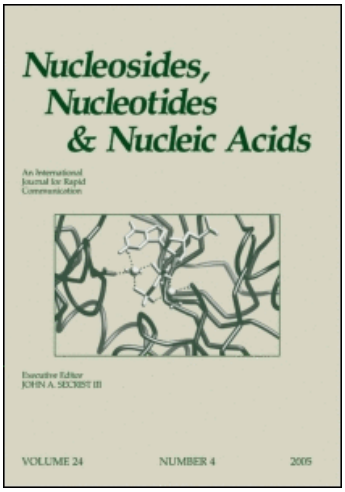

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IMMUNOCYTOCHEMICAL DETECTION OF hENT1 and hCNT1 IN NORMAL TISSUES, LUNG CANCER CELL LINES, AND NSCLC PATIENT SAMPLES

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□ *Nucleoside transporters are essential for the cellular entry, efficacy, and cytotoxicity of several clinically important deoxynucleoside analogs (e.g., cytarabine and gemcitabine). We used immunohistochemistry to determine protein expression levels of the nucleoside transporters hENT1 and hCNT1 in NSCLC cell lines, NSCLC patient samples, and a variety of normal tissues. All 4 NSCLC cell lines expressed high to very high levels of both hENT1 and hCNT1. In NSCLC and normal tissues expression of hENT1 and hCNT1 ranged from completely negative to high. Immunohistochemistry might be a useful tool to predict response to deoxynucleoside analogs in malignancies treated with these drugs.*

Keywords Human equilibrative nucleoside transporters (hENTs); human concentrative nucleoside transporters (hCNTs); deoxynucleoside analogs; antimetabolite cytotoxic drugs; immunocytochemical staining

INTRODUCTION

Deoxynucleoside analogs (NAs) form a major group of antimetabolite cytotoxic drugs in current clinical use. Cytarabine (ara-C) for instance is the most effective agent in the treatment of acute myeloid leukemia (AML) and gemcitabine is used extensively for the treatment of solid tumors.[1] Two groups of nucleoside transporters (human equilibrative nucleoside transporters, hENTs, and concentrative nucleoside transporters, hCNTs) mediate cellular uptake of NAs by facilitated diffusion or concentrative

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uptake.[2] NAs differ with respect to their preferential transporters, hENT1 for instance is responsible for 80% of the influx of ara-C.[3,4] NAs require active nucleoside transport into the cell in order to exert their cytotoxicity.

The aim of our study was to set up an immunostaining procedure in order to determine the protein expression of hENT1 and the pyrimidine-prefering hCNT1 transporter in normal tissues, non-small-cell-lung-cancer (NSCLC) cell lines, and patient samples.

MATERIALS AND METHODS

Cell Lines

Four NSCLC cell lines were used in this study: A549 (human alveolar basal epithelial cells); H460 (human pleural effusion; adenocarcinoma); H292 (human lung carcinoma); and SW1573 (human lung cancer). SW1573 cells were cultured in DMEM medium supplemented with 10% FCS and 20 mM HEPES. The other NSCLC cell lines were cultured in RPMI medium containing 10% FCS and 20 mM HEPES.[5] Cytospins were prepared by centrifugation of 50 μl of cell suspension (0.5×10^6 cells/ml PBS containing 5% human serum albumin) at 50 g for 7 minutes. Cytospins were air-dried on silica gel for at least 48 hours and subsequently stored at –20°C.

Normal Tissues

Tissue microarrays (TMAs) were prepared containing the following normal tissues: heart, lung, tonsil, spleen, thymus, kidney, bladder, skin, placenta, cerebrum, cerebellum, muscle, bone marrow, breast, nerve, liver, oesophagus, stomach, gall bladder, colon, small intestine, colon, adrenal gland, thyroid, testis, ovary, uterus, cervix, neurohypophysis, adenohypophysis, pancreas, and prostate. Each slide contained three spots from each tissue.

Patient Samples

Formalin-fixed paraffin-embedded tumor samples from five patients with NSCLC were evaluated. Tissue sections were cut in 4-μm sections and mounted on poly-L-lysine-coated slides. Slides were stored at room temperature.

Immunocytochemistry

Immunocytochemical staining was performed on (cryo-preserved) cytospins using a standard alkaline phosphatase/anti-alkaline phosphatase (APAAP) method. Cells were fixed with acetone (10 minutes RT). Slides were washed with PBS (2×5 minutes) and incubated overnight with

the primary antibodies, rabbit-anti human hENT1 and hCNT1[6] diluted 1:100 and 1:150, respectively, in PBS/1%BSA/0.1%Na-azide. The following day, slides were washed (2 × 5 minutes in PBS) and incubated with the secondary antibody, biotinylated swine-anti-rabbit (Dako, Glostrup, Denmark) diluted 1:300 in PBS/1%BSA/2%PHS/0.1%Na-azide for one hour. After wash steps, slides were incubated with the alkaline-phosphatase conjugated streptavidine (1:100 in PBS/1%BSA/0.1%Na-azide, 30 minutes; Dako, Glostrup, Denmark). Visualization of AP was performed by incubation in New Fuchsin/naphtol ASBI phosphate solution supplemented with levamisole. Cells were counterstained with Mayer's Haematoxylin Solution (Merck, Darmstadt, Germany) and embedded in Aquamount.

Immunohistochemistry

TMAs containing normal tissues and NSCLC tissue sections were deparaffinized and rehydrated by incubation in 100% xylene (2 × 10 minutes), followed by incubation in decreasing ethanol concentrations. Sections were fixed and endogenous myeloperoxidase was blocked (methanol/0.3% H_2O_2, 30 minutes). Antigen retrieval was performed by heating the slides in a microwave for 15 minutes in 10 mM citrate buffer solution (pH 6.0). Slides were cooled down at room temperature for at least 30 minutes and washed (PBS). Nonspecific staining was blocked using normal swine serum (1:10; 10 minutes). Slides were incubated with the primary antibodies, rabbit-anti human hENT1 and hCNT1 diluted 1:100 and 1:150, respectively, in PBS/1%BSA/0.1%Na-azide for 2 hours. After PBS wash steps, the secondary antibody, biotinylated swine anti-rabbit (1:300 diluted in PBS/1%BSA/2%PHS, 30 minutes; DAKO, Glostrup, Denmark) was applied. Slides were rinsed (PBS) and incubated with horseradish peroxidase-conjugated streptavidin (1:100 in PBS/1%BSA, 45 minutes; DAKO, Glostrup, Denmark). Peroxidase activity was determined using 1 mM 3,3′ diaminobenzidine (DAB, Sigma, St. Louis, MO, USA), 0.05 M imidazole (Merck, Darmstadt, Germany) and 0.036% H_2O_2 in 0.05M Tris Buffer (pH 7.4) for 10 minutes. Cells were counterstained using Mayer's Haematoxylin Solution (Merck, Darmstadt, Germany) and embedded in malinol.

Negative controls were performed by omitting the first antibody. hENT1 and hCNT1 protein expression were evaluated by two independent investigators by scoring the intensity of the staining: negative (−), low (−/+), high (+), or very high (++).

Taqman Analysis

Analysis of hENT1 expression in the NSCLC cells was performed by real-time PCR with the Applied Biosystems 7500HT sequence detection

system (Applied Biosystems, Foster City, CA, USA).[7] Primers and probes for hENT1 (NM_004955) and the housekeeping gene β-actin were obtained from Applied Biosystems Assay-on-Demand products. PCR reactions were performed in triplicate using 5 ml of cDNA, 12.5 ml of TaqMan Universal PCR Master Mix, 2.5 ml of probe and 2.5 ml of forward and reverse primers in a final volume of 25 ml. Quantification of gene expression was referred to standard curves obtained with dilutions of cDNA obtained from Quantitative PCR Human Reference Total RNA (Stratagene, La Jolla, CA, USA).

RESULTS

Immunohistochemical staining demonstrated expression of hENT1 and hCNT1 in several different tissues (Table 1). hENT1 was not expressed in heart, tonsil, spleen, thymus, bladder, placenta, muscle, ovary, uterus, and neurohypophysis tissue. Expression of hENT1 was low in the following tissues: lung, cerebrum, nerve, oesophagus, stomach, gall bladder, colon, small intestine, thyroid, testis, uterus, cervix, pancreas, and prostate. A high expression of hENT1 was observed in adrenal gland, kidney, skin, bone marrow, breast, liver, and adenohypophysis tissue. We did not detect hCNT1 in tonsil, spleen, thymus, bladder, muscle, bone marrow, gall bladder, testis, ovary, uterus, neurohypophysis, and prostate tissue. HCNT1 expression was low in heart, lung, placenta, cerebrum, nerve, liver, esophagus, stomach, colon, small intestine, thyroid, uterus, cervix, adenohypophysis, and pancreas tissue. Breast and kidney tissue expressed high levels of hCNT1 protein.

All four NSCLC cell lines expressed both hENT1 and hCNT1 (Table 2). hENT1 and HCNT1 expression were high in the A549 and SW1573 cells. H292 and H460 cells expressed very high levels of hENT1 and hCNT1. In the samples from NSCLC patients, expression of hENT1 and hCNT1 ranged from completely negative to high (Table 2). We also determined hENT1 mRNA expression (ratio over β-actin) in the NSCLC cell lines by Taqman analysis (Figure 1). H460 (0.38 ± 0.04) and H292 (0.36 ± 0.05) cells expressed higher levels of hENT1 mRNA than A549 (0.26 ± 0.002) and SW1573 (0.27 ± 0.02) cells. hENT1 mRNA levels therefore appear to be consistent with hENT1 protein expression in these NSCLC cell lines.

DISCUSSION

Several clinically important NAs depend on transport by plasma membrane nucleoside transporters such as hENT1 and hCNT1 for cellular entry. These nucleoside transporters can therefore be the rate-limiting step in the efficacy and cytotoxicity of NAs[2]. In AML for instance, *hENT1* mRNA expression has been related to *in vitro* drug resistance and outcome.[8–10]

TABLE 1 hENT1 and hCNT1 expression in normal tissues

Tissue	*h*ENT1	*h*CNT1
Heart	−	+/−
Lung	+/−	+/−
Tonsil	−	−
Spleen	−	−
Thymus	−	−
Kidney	+	+
Bladder	−	−
Skin	+	+
Placenta	−	+/−
Cerebrum	+/−	+/−
Cerebellum	+	+
Muscle	−	−
Bone marrow	+	−
Breast	+	+
Nerve	+/−	+/−
Liver	+	+/−
Oesophagus	+/−	+/−
Stomach	−	−
Stomach (ant)	+/−	+/−
Gall bladder	+/−	−
Colon	+/−	+/−
Small intestine	− to +/−	− to +/−
Adrenal gland	+	+/−
Thyroid	− to +/−	− to +/−
Testis	+/−	−
Ovary	−	−
Uterus	−	−
Uterus	+/−	+/−
Cervix	− to +/−	+/−
Neurohypophysis	−	−
Adenohypophysis	+	+/−
Pancreas	+/−	+/−
Prostate	+/−	−

TABLE 2 Expression of hENT1 and hCNT1 in NSCLC cell lines and patient samples

	*h*ENT1	*h*CNT1
Cell lines		
A549	+	+
H460	++	++
H292	++	++
SW1573	+	+
Patient samples NSCLC		
1	−	−
2	+/−	+/−
3	+/−	+/−
4	+/−	+/−
5	+	+/−

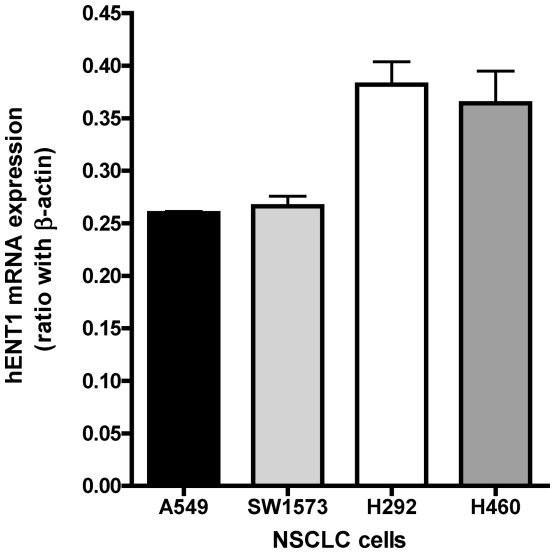

FIGURE 1 hENT1 mRNA expression (ratio over β-actin) in NSCLC cell lines determined byTaqman analysis.

In NSCLC, pancreatic adenocarcinoma and biliary tract carcinoma hENT1 mRNA expression was associated with sensitivity to gemcitabine.[11,12] It is therefore important to know the tissue distribution and the expression level of nucleoside transporters in tumors as well as in normal tissues; we therefore stained several normal tissues, NSCLC cell lines and patient samples for hENT1 and hCNT1 protein expression. We observed a wide range of hENT1 and hCNT1 expression in the normal tissues. Most tissues expressed both transporters. All four NSCLC cell lines expressed both hENT1 and hCNT1. hENT1 expression in these cell lines correlated with mRNA levels as determined by Taqman analysis. One NSCLC patient sample did not express hENT1 or hCNT1, while the other four patient samples expressed both transporters. The absence of hENT1 expression was recently reported to predict nonresponse to gemcitabine-containing chemotherapy in NSCLC.[13] Immunocytochemical detection could therefore be a valuable tool for pre-treatment screening of transporter levels in malignancies to be treated with NAs, because this method requires only a small number of tumor cells, while cellular morphology is maintained allowing studies on the localization of hENT1 and hCNT1.

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