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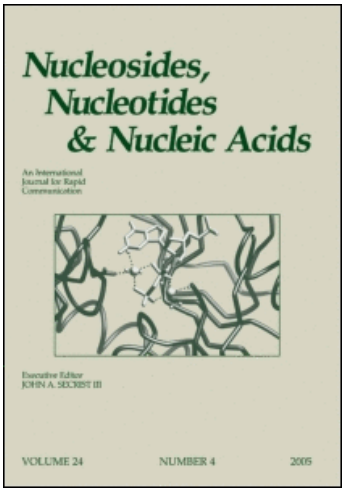

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Human Nucleoside Transporters: Biomarkers for Response to Nucleoside Drugs

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HUMAN NUCLEOSIDE TRANSPORTERS: BIOMARKERS FOR RESPONSE TO NUCLEOSIDE DRUGS

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□ *This review describes recent advances in developing human nucleoside transporters (hNTs) as biomarkers to predict response to nucleoside analog drugs with clinical activity. Understanding processes that contribute to drug response or lack thereof will provide strategies to potentiate efficacy or avoid toxicities of nucleoside analog drugs. hNT abundance, evaluated by immunohistochemical methods, has shown promise as a predictive marker to assess clinical drug response that could be used to identify patients who would most likely benefit from nucleoside analog drug treatment.*

Keywords Nucleoside transporters; anticancer nucleoside drugs; immunohistochemistry; biomarkers; clinical response

INTRODUCTION

Nucleoside analog chemotherapy is used widely to treat hematological and solid tumors. Human nucleoside transporters (hNTs) mediate membrane transport of physiologic and therapeutic nucleosides and hence are pharmacological determinants of drug bioavailability and response to therapy.[1,2] Plasma membrane transport of hydrophilic nucleosides in humans is mediated by two specialized protein families, equilibrative hNTs (hENTs) and concentrative hNTs (hCNTs), both of which were

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identified by molecular cloning and functional expression of cDNAs encoding various NT family members.[3,4] Anticancer clinical nucleoside drugs include both purine and pyrimidine nucleoside analogs such as fludarabine (9-β-D-arabinofuranosyl-2-fluoroadenine), cladribine (2-chloro-2′-deoxyadenosine), clofarabine (2-chloro-9-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl) adenine), cytarabine (1-β-D-arabinofuranosylcytosine), gemcitabine (2′-deoxy-2′,2′-difluorocytidine), and capecitabine (5′-deoxy-5-fluoro-*N*-[(pentoxy)carbonyl]-cytidine). Although all nucleosides enter cells in small quantities by passive diffusion, these anticancer nucleoside analog drugs enter human cells primarily via hNTs and are subsequently metabolized intracellularly and incorporated into DNA (and/or RNA) resulting in chain termination, apoptosis and cell death. In some patients, partial or complete response is achieved with anticancer nucleoside drugs whereas in others little or no response is observed. The variability in response is multifactorial with the first step in achieving cytotoxicity being membrane permeation. The presence of functional NTs in plasma membranes has been shown to be required for cytotoxicity of most nucleoside drugs. Lack of functional NTs has been correlated with drug resistance in cultured cancer cell lines, giving rise to the suggestion that low abundance of NTs in target cancer cells would give rise to resistance in patients.[5] Development and validation of biomarkers to predict tumor accumulation of anticancer nucleoside drugs, which in turn would predict response to therapy, should improve efficacy of anticancer nucleoside drugs.

This review focuses on roles of hNTs in uptake of therapeutic nucleoside drugs and development and use of anti-hNT antibodies to evaluate hNTs abundance as predictive markers to individualize therapy.

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Role of hNTs in Permeation of Anticancer Drugs

hENTs and hCNTs play key roles in nucleoside and sometimes nucleobase uptake and thus are key determinants of efficacy of many nucleoside drugs used in anticancer or anti-viral therapies. hENTs mediate equilibrative bi-directional transport of nucleosides across membranes,[6,7] whereas hCNTs transport nucleosides against their concentration gradients driven by sodium and/or proton coupled electro-chemical gradients.[8] To date, four hENT and three hCNT subtypes (hENT1–4, hCNT1–3) have been identified by molecular cloning and functional expression of their cDNAs.[6,9–14]

hENT1 and hENT2, which are found primarily in plasma membranes, are distinguished, respectively, by their high and low sensitivities to inhibition by a potent and highly specific transport inhibitor nitrobenzylmercaptopurine ribonucleoside (NBMPR).[10,11,15] They both transport purine and pyrimidine nucleosides and hENT2 also transports some nucleobases.[16] hENT3 is found in intracellular membranes, and exhibits broad permeant

selectivity whereas hENT4, which is found in plasma membranes, transports adenosine and monoamines in brain and heart.[6,17,18] hCNTs all transport uridine but exhibit different preferences for other permeants, with selectivities by hCNT1 for pyrimidine nucleosides, hCNT2 for purine nucleosides and hCNT3 for both purine and pyrimidine nucleosides.[12–14]

NTs are regulated proteins and most cells possess more than one NT subtype, thus exhibiting the capacity for uptake of a broad range of nucleosides. In cultured cancer cells, hENT1 protein levels increase between G1 and G2-M phases of the cell cycle[19,20] and higher proliferation rates are associated with higher abundance of hENT1 protein.[21,22] Increases in hENT1 protein were shown in patients undergoing leukapheresis, [23] cytotoxic nucleoside,[24] or granulocyte-macrophage colony-stimulating factor[24] treatments. NTs, which play key roles in salvage pathways for nucleotide synthesis, are broadly distributed in cells and tissues and thus are involved in cellular uptake and cytotoxic activity of many anticancer nucleoside drugs.

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Clinical Nucleoside Drugs: Correlation Between Transportability, mRNA, and/or Protein Levels and Chemotherapeutic Sensitivity

Fludarabine and cladribine are used to treat chronic lymphocytic leukemia (CLL).[25] Cladribine is active in both CLL and hairy cell leukemia (HCL).[26] Both drugs have activity towards low-grade non-Hodgkin's lymphoma (NHL), Waldeström's macroglobulinaemia (WM) and other hematological malignancies but little activity in solid tumors.[27] Fludarabine cytotoxicity to leukemia cells was positively correlated with hENT1 abundance by flow cytometry using a fluorescent hENT1 probe.[28] In addition, a positive correlation between hENT2 protein levels and fludarabine sensitivity was also shown in CLL cells.[29] Sensitivity to cladribine was greater in cells with high hENT1 abundance in acute lymphoblastic leukemia (ALL) cells.[28] Clofarabine is a new-generation purine nucleoside drug with structural similarities to fludarabine and cladribine that exhibits activity towards colon and renal cancers, pediatric and adult ALL and myelodysplastic syndrome (MDS).[30] Cellular uptake of clofarabine, like that of fludarabine and cladribine, is mediated by hENT1, hENT2, hCNT2, and hCNT3[31,32] and it is therefore likely that clofarabine sensitivity will also be associated with hENT1 abundance.

Several pyrimidine nucleoside analog drugs, including cytarabine, gemcitabine and capecitabine, are used to treat cancer. Cytarabine is effective in acute myeloid leukemia (AML) and its influx in human leukemic blast cells is mediated mainly by hENT1 and to a smaller extent by hENT2.[2,33] In samples from 55 cytarabine sensitive AML patients, *hENT1* mRNA expression was positively correlated with sensitivity to cytarabine.[34] In this study, expression of the *hENT1* gene was the most important factor

for determining cytarabine sensitivity and samples that exhibited in vitro resistance to cytarabine had *hENT1* mRNA levels that were one-third of those from samples that exhibited in vitro sensitivity. In a study of *hENT1* mRNA expression levels in adult AML patients,[35] lower levels were associated with shorter disease-free survival (DFS). High levels of *hENT1* mRNA in infants with *MLL* gene-rearranged ALL[36] were associated with high cytarabine sensitivity. These studies have laid the foundation for *hENT1* mRNA levels to be used as a predictor of sensitivity to cytarabine (or possibly to other deoxynucleoside analogs) at diagnosis.

Gemcitabine has activity in bladder, non-small cell lung, ovarian, pancreatic, breast, head and neck cancers.[2] Gemcitabine enters cells via hENT1, hENT2, hCNT1, and hCNT3.[37,38] Cultured human cell lines that are deficient in nucleoside transport either pharmacologically (through inhibition by NBMPR, a high affinity inhibitor of hENT1) or genetically (through selection of variants resistant to cytotoxic nucleosides) exhibit resistance to gemcitabine,[39] suggesting that hENT1 abundance may be used as a predictive marker for response to gemcitabine. Consistent with this proposal, *hENT1* mRNA expression correlated positively with gemcitabine sensitivity in non-small-cell lung cancer cell lines.[40] In other studies, transcription analysis of *hENT1* and several other genes involved in gemcitabine metabolism showed that expression of *hENT1* mRNA was the most important predictor of gemcitabine response in pancreatic cancer patients treated with gemcitabine,[41] thus suggesting that *hENT1* mRNA is a potential biomarker for gemcitabine response in patients. In 12 bladder cancer patients treated with intravesicle gemcitabine, *hENT1* mRNA levels were higher in patients who had a complete response.[42]

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Capecitabine, an oral prodrug of 5-fluorouracil, is rapidly converted to 5′-deoxy-5-fluorocytidine by liver carboxylesterase and then sequentially to 5′-deoxy-5-fluorouridine by cytidine deaminase in liver and tumors and to 5-fluorouracil by thymidine phosphorylase, which is present in high abundance in tumor tissues compared to normal tissues.[43] Capecitabine has activity in renal, prostate, pancreatic, and ovarian cancers and is currently used in patients with metastatic colorectal cancer.[44] Although capecitabine itself is not a permeant for any of the hNTs, its metabolite, 5′-deoxy-5-fluorouridine, is a permeant for hENT1, hCNT1, and hCNT3.[45,46] Blocking hENT1 transport activity in primary breast cancer cells by NBMPR resulted in significant protection against cytotoxic effects of capecitabine, showing a role of hENT1 in capecitabine cytotoxicity.[47]

Although many nucleoside drugs are permeants for multiple hNTs, hENT1 is the only hNT that has been associated with therapeutic response to a particular drug in patients. This is believed to be due to the apparently ubiquitous presence of hENT1 in cells and tissues predominantly on plasma membranes (thus facilitating entry of nucleoside drugs into cells) compared to the hCNTs, which have relatively narrow tissue distributions. Al-

though hCNTs are concentrative and thus have the potential of enhancing therapeutic response by increasing drug uptake in tumor versus normal tissues, some studies reported the presence of intracellular hCNT,[48] which was postulated to be one of the reasons for lack of correlation between hCNT abundance and therapeutic response. In cultured cells, increased sensitivity to fluoropyrimidine nucleosides was observed with acquisition of hCNT1 function in transfected cells,[39,49] presumably because of the increase in hCNT1 in plasma membranes. It has also been suggested that high levels of hCNT can overcome the inhibitory action of antimetabolites of de novo synthesis by enhancing the salvage of nucleosides.[50] In addition, expression of other genes that mediate nucleoside drug resistance and prior treatment with other cytotoxic drugs may alter response to nucleoside drugs.

Impact of Genetic Polymorphisms on Nucleoside Drug Efficacy

Genetic variants (single nucleotide polymorphisms, SNPs) of the *hENT* and *hCNT* genes in ethnically diverse populations have been studied in recent years to determine if genetic variations are responsible for clinical variability in response to nucleoside drugs. In studies of the *hENT1* gene, it was concluded that coding region variants do not contribute to inter-individual differences in response to nucleoside drugs.[51] However, a recent investigation found three SNPs in *hENT1*'s promoter region and individuals with the CGG/CGC haplotype showed 1.37-fold higher median expression of *hENT1* mRNA than those with common CGG/CGG haplotypes, thus suggesting that promoter region SNPs may influence gene expression and thus alter cytarabine chemosensitivity.[52] Fourteen SNPs, including 11 in the coding region, were found in the *hENT2* gene at very low frequencies, and five of the altered proteins as well as the reference hENT2 were able to transport the test nucleosides (inosine, guanosine) with the exception of ENT2-Δ845–846, which resulted in a truncated protein due to a frameshift mutation.[53]

In the *hCNT1* gene, 58 SNPs were identified in exons and introns, thus revealing that *hCNT1* is a highly variable gene.[54] In functional studies using *Xenopus* oocytes, all variant proteins transported thymidine with the exceptions of hCNT1-S546P, hCNT1–1153del, and hCNT1-V189I, exhibited reduced affinity for gemcitabine. Studies of interaction of gemcitabine with CNT1 and four of the most common altered protein variants demonstrated that gemcitabine exhibited 50% reduced potency in interacting with CNT1-Val189Ile compared with the reference CNT1. These results are important in the context of pharmacokinetic concentration and therapeutic efficacy of gemcitabine.

In the *hCNT2* gene, ten coding region variants were identified of which six non-synonymous variant proteins transported guanosine with no significant differences observed in comparison with reference hCNT2 with

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the exception of CNT2-S75R, the coding region of which was ligated into a different cloning vector than the other variants and thus was not experimentally equivalent. Among the four synonymous variant proteins, hCNT2-F355S exhibited altered preferences for inosine and uridine (increase and decrease, respectively) compared to those of reference hCNT2.[55] In a more recent study, genetic variability of the *hCNT2* gene in three ethnic populations identified 23 SNPs in exonic and intronic regions[56] and differences in frequencies were evident among the three ethnic groups. In 270 DNA samples from U.S. populations,[57] 56 SNPs in the exons and introns of the *hCNT3* gene were identified, and all non-synonymous variant proteins, except one, had transport properties that were similar to those of the wild type protein. A parallel study using 96 DNA samples revealed 16 *hCNT3* variants, of which five were nonsynonymous variants in coding regions, although there were no observed differences in activity or protein abundance.[58]

Low allele frequencies of various SNPs (<1 SNP per 100 base pairs) and lack of functional effects in the non-synonymous coding SNPs in the *hENT1, hENT2,* and *hCNT3* genes imply high conservation of function among these genes. The high frequency of *hCNT1* variants (1.5 SNPS per 100 base pairs) relative to that of *hCNT2* variants (1 SNP per 100 base pairs) suggests that the *hCNT1* gene is more variable than the *hCNT2* gene. Whether or not genetic variations in hNT genes contribute to variation in uptake and disposition of clinically used nucleoside analogs is unclear at this time.

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Correlation of hENT1 Protein Abundance to Clinical Response to Nucleoside Drugs

Several in vitro studies[5,28,34,39,40,59–62] have demonstrated a correlation between abundance of hNTs and cytotoxicity of nucleoside analog drugs. The initial assays for quantifying hNT protein levels relied on high-affinity binding of radio-labeled or fluorescent NBMPR to cells,[10] a technique that is not suitable for solid tumors. Monoclonal antibodies specific for the hENT1 protein raised against a synthetic peptide comprised of residues 254–271 from the large intracellular loop of hENT1 have been developed and validated in a variety of immunologic assays by standard protocols.[47,63]

Immunohistochemical staining of tissue samples is routinely used to evaluate the presence of biomarkers. Mackey et al.[47] developed an immunohistochemical method to assess hENT1 abundance in cancer tissues, demonstrating a positive correlation between increased numbers of NBMPR binding sites and hENT1 staining intensities.[47] Marked intertumoral variability was noted in hENT1 staining intensities among invasive adenocarcinoma cells from 33 primary breast tumors.[47] Similarly, immunohistochemistry with monoclonal antibodies against hENT1 was used to assess hENT1 abundance in frozen sections of lymph nodes from 30

patients with Hodgkin's disease (HD), demonstrating that hENT1 staining varied among Reed-Sternberg cells from different HD samples (64). In a recent study of NHL patients, lymph nodes of 115 cases of various subtypes and 15 controls were evaluated for the presence of hENT1 protein using immunohistochemistry; a high frequency of hENT1 staining was found in Burkitt lymphoma/leukemia, diffuse large B-cell lymphoma and follicular lymphoma.[65] High hENT1 mRNA and protein levels were demonstrated in Mantle cell lymphoma cells (MCL) compared to CLL cells, MCL cells being more sensitive to nucleoside analog drugs.[66] Observation of this variability between and within tumor types provided the impetus to further study hENT1 immunohistochemistry as a marker of prognosis and clinical outcomes in patients treated with nucleoside drugs.

In a retrospective study[67] of gemcitabine-treated pancreatic patients, immunohistochemical staining for hENT1 abundance was demonstrated to be a prognostic marker for survival after gemcitabine therapy. Patients with detectable hENT1 staining had a significantly longer survival than patients with no hENT1 staining (13 months vs. 4 months). Consistent with this result, patients with high levels of hENT1 mRNA exhibited an increase in overall survival (OS), DFS and time to progression in gemcitabine-treated pancreatic cancer.[41] Recent in vitro studies in models with either native or over-expressed recombinant hENT1 demonstrated that levels of hENT1 mRNA and/or protein are correlated with gemcitabine response in pancreatic adenocarcinoma and biliary tract carcinoma cells.[68,69]

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Using hENT1 immunohistochemistry, Seve et al.[70] demonstrated that only 16% of 43 non-small cell lung cancer (NSCLC) samples were positive for hENT1. Gemcitabine is a widely used drug in NSCLC, and a recent study demonstrated the usefulness of hENT1 staining in biopsy samples in 24 NSCLC samples before application of chemotherapy with the absence of hENT1 staining predicting non-response.[71]

To formally evaluate hENT1 immunohistochemistry as a predictive marker for benefit from gemcitabine or other nucleoside therapy, correlative studies are required in populations from clinical trials in which patients are randomized to treatment with or without nucleoside drugs. Results of such a study were recently reported.[72] The predictive value of hENT1 abundance was examined in a cohort of 538 patients with pancreatic adenocarcinoma. In this Radiation Therapy Oncology Group (RTOG) 9704 prospective randomized trial of gemcitabine-treated patients, a statistically significant association between higher hENT1 abundance and longer OS was demonstrated relative to that of patients with low hENT1 abundance. These results suggest that high hENT1 abundance predicts benefit from gemcitabine in patients with resected pancreatic cancer and further strengthens the hypothesis that hNTs are determinants of therapeutic response to nucleoside drugs.

In a recent study[73] the abundance and distribution of hENT1 was assessed in tumor tissues from 41 patients with radically resected cancer of the ampulla to identify patients with a likelihood of benefiting from gemcitabine chemotherapy. In this cohort of patients nearly one-third had high hENT1 staining, suggesting that these patients may benefit from gemcitabine therapy.

The Predictive Value of Other hNTs

In an analysis of tissue microarrays for the presence of NTs by immunohistochemistry in 300 gynecologic tumors,[74] it was shown that the majority of tumors were positive for hENT1. Furthermore, most of the NT negative ovarian tumors (i.e., lacking hENT1, hENT2, and hCNT1) were clear cell carcinomas and most of the hCNT1 negative tumors were adenocarcinomas, thus showing that the NT phenotype was related to tumor histology with the absence of hCNT1 being more frequent than that of hENT1 or hENT2 and low hCNT1 protein being highly correlated with poor prognosis. Fludarabine is used to treat CLL and the role of hENT2 in response to fludarabine in CLL patients was investigated in cells isolated from 21 patients with CLL, showing a significant positive correlation between hENT2 levels, as measured by immunoblotting, and sensitivity to fludarabine cytotoxicity.[29] In a cohort of 90 breast cancer patients who underwent surgery and cyclophosphamide-methotrexate-5-fluorouracil therapy, the presence of hCNT1 staining, which was mostly cytoplasmic with some nuclear staining, was suggested to have a negative prognostic value for DFS and risk of relapse.[75] A higher percentage of hCNT1-positive cells correlated with lower DFS and higher risk of relapse. One of the reasons for the inverse relationship between hCNT1 positivity and DFS could be greater uptake of thymidine (i.e., rescue from methotrexate toxicity) in cells with higher surface hCNT1. Although hCNT1 is primarily a plasma membrane transporter, it has also been observed inside cells in some epithelial tissues.[76] The presence of hNTs, if located intracellularly rather than on plasma membranes, may not predict therapeutic benefit with nucleoside drugs.

In studies involving 56 CLL patients,[48] the most significant indicator of clinical fludarabine resistance was high expression of *hCNT3* mRNA whereas *hENT1* and *hENT2* mRNA levels were not associated with fludarabine response. This was an unexpected finding, since no hCNT3-mediated cellular uptake of fludarabine was detected in CLL samples, and high hCNT3 would be expected to confer drug sensitivity if present on the plasma membrane of cells thus allowing entry of cytotoxic drugs into cells. Immunohistochemistry with anti-hCNT3 antibodies revealed cytoplasmic staining consistent with an intracellular location of hCNT3 in CLL, and the mechanism of the resulting resistance may be explained by the lack of cell surface hCNT3. To

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further explore hCNT3 immunohistochemistry as a marker for fludarabine resistance in CLL, tissue samples from 36 patients who were treated with fludarabine were stained with anti-hCNT3 monoclonal antibodies, and a strong relationship between hCNT3 staining and clinical resistance to fludarabine was observed.[77] These findings suggest that hCNT3 may have a role in cellular resistance to fludarabine, and although the exact mechanism of this resistance is not known, it may be due to lack of cell surface hCNT3 activity. The potential for hCNT3 staining as a predictive tool in the treatment of fludarabine-based therapies warrants further study.

The prognostic value of determining both hENT1 and hCNT3 protein abundance in resected pancreatic cancer patients with gemcitabine monotherapy followed by concomitant radiotherapy and gemcitabine treatment was evaluated in a recent study by Maréchal et al.[78] hENT1 and hCNT3 were evaluated in tumor samples obtained from 45 patients by immunohistochemistry using monoclonal antibodies, respectively, against hENT1 and hCNT3. Patients with high hENT1 had higher median DFS than patients with low hENT1 (46.8 vs. 8.4 months) and patients with high hCNT3 had higher median DFS than patients with low hCNT3 (23.5 vs. 8.6 months). In patients with high hENT1 and high hCNT3, two favorable markers, the mean OS time was 94.8 months compared to 12.2 months for patients with no favorable markers.

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CONCLUSIONS

High hENT1 levels are positively correlated with response to gemcitabine in pancreatic and lung cancers, suggesting that hENT1 can be used as a predictive marker for response to gemcitabine in these cancers However, studies in lymphoma[79] suggest that high hENT1 abundance may be associated with shorter survival and poorer response in other cancer types. However, the latter study involved chemotherapy with three agents (gemcitabine, vinorelbine, and doxorubicin) and the relationship between gemcitabine efficacy and hENT1 abundance may have been altered by the other two agents. A role for hCNT3 in prediction of gemcitabine sensitivity was shown recently[78] and the authors established the prognostic value of a combination of high hENT1 and high hCNT3 abundance with better response and OS. However, in other studies, high levels of hCNT1 and hCNT3 were associated with poor response to nucleoside drugs. Thus, although NT proteins, particularly hENT1 and hCNT3, are important determinants of response to nucleoside drugs, other factors, including cancer type, the presence of various nucleoside-metabolizing enzymes, DNA damage repair mechanisms and prior treatment with other chemotherapy agents that result in up or down-regulation of NT proteins may also have to be considered in future analyses.

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