ABSTRACT

SHANNON CUMMINS
Diminution of Concentrative Nucleoside Transporter 1 (CNT1) Activity in Human Ovarian Cancer Cells: Subtype-Dependent Gemcitabine Response to Exogenously Expressed hCNT1
(Under the direction of DR. RAJGOPAL GOVINDARAJAN)

Human concentrative nucleoside transporter1 (hCNT1), a member of the solute carrier 28 family, is a high-affinity transporter of anti-cancer nucleoside drugs (e.g. gemcitabine). While hCNT1 is implicated in sensitizing solid tumors to gemcitabine, its role in determining gemcitabine efficacy in human ovarian cancers remains unknown. Here we examined the functional expression of hCNT1 in normal and cancerous ovarian cells and compared its contributions towards gemcitabine efficacy in histological subtypes of ovarian cancer. RNA and protein analysis identified that unlike normal ovarian surface epithelial cells (IOSE80), which expressed high levels of hCNT1 at the cell surface, hCNT1 expressions were diminished in ovarian cancer cell lines. Consistently, ^{3}H-gemcitabine transports in ovarian cancer cells were reduced by 3-10 fold compared with that of IOSE80 cells. Stable retroviral expression of hCNT1 in various ovarian cancer cell lines displayed variations in hCNT1 localizations, ^{3}H-gemcitabine transports, and drug sensitivities. hCNT1-expressing endometroid cancer cells exhibited the highest sensitivity to gemcitabine treatment (~140 fold IC_{50} decrease). hCNT1-expressing serous ovarian cancer cells (OV90) displayed a moderate level of sensitivity (~25 fold IC_{50} decrease) that was ~2000-fold higher than that observed with clear cell carcinoma (CCC; ES-2 and TOV21G) and teratocarcinoma cells (PA-1). hCNT1 was not recruited to the cell surface in teratocarcinoma cells and was mistargeted to golgi in CCC cells. These data show that hCNT1 transportability is highly diminished in human ovarian cancers and the reintroduction of hCNT1 can selectively regain gemcitabine sensitivity in the endometroid and serous subtypes but not in the teratocarcinoma or CCC subtypes.

INDEX WORDS: Thesis, Honors Program, The University of Georgia, Ovarian Cancer, Nucleoside Transporters, Gemcitabine

DIMINUATION OF CONCENTRATIVE NUCLEOSIDE TRANSPORTER 1 (CNT1) ACTIVITY IN HUMAN OVARIAN CANCER CELLS: SUBTYPE DEPENDENT GEMCITABINE RESPONSE TO EXOGENOUSLY EXPRESSED HCNT1

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CHAPTER ONE
INTRODUCTION

Ovarian cancer is the fifth leading cause of cancer-related death in women worldwide, and it also has the highest mortality rate among all cancers of the female reproductive system.[1] One major problem typically associated with ovarian cancer is that there are few early symptoms and no proven screening tests, which leads to diagnosis at a later, more advanced stage.[1] In 2006, the FDA approved 2',2' difluorodeoxycytidine (dFdC; gemcitabine) to be used against ovarian cancer in those who had advanced stages of ovarian cancer or who had shown initial resistance to conventional chemotherapeutic agents (e.g. carboplatin).[2] In the initial trial, dFdC administered with carboplatin was shown to improve progression-free survival from 5.8 months to 8.6 months.[2] In spite of that, those taking gemcitabine displayed higher levels of toxicity and the overall survival was not increased by the use of gemcitabine; it averaged around 18 months for both sections of the study.[3] Reports have surfaced regarding gemcitabine resistance in patients, where the tumor cells are nonresponsive to gemcitabine.[4-7] Although gemcitabine improves benefits for many patients, its total effects are modest, and a portion of patients will have no response at all. By specifically identifying the mechanism of gemcitabine activity, it would be possible to alter the activity of the drug in order to improve the sensitivity and decrease levels of resistance.

In order for gemcitabine to be effective, it must first enter the tumor cells and be activated by phosphorylation. Gemcitabine is a hydrophilic nucleoside analog structurally similar to cytidine, and thus can enter the cell through nucleoside transporters that move preformed nucleosides from the extracellular space into the cell for DNA replication. There are two types of

nucleoside transporters: equilibrative nucleoside transporters (ENTs) and concentrative nucleoside transporters (CNTs). Among all CNTs and ENTs, four members are capable of transporting gemcitabine into the cell: ENT1, ENT2, CNT1, and CNT3. When gemcitabine enters the cell through a nucleoside transporter, it is then phosphorylated to its diphosphate (dFdCDP) and triphosphate (dFdCTP) forms and incorporated into the DNA, thus inhibiting DNA synthesis and inducing cell death.[4] In cells with resistance to gemcitabine, either the initial entry into the cell via the nucleoside transporters or the phosphorylation may be inhibited.[4] Deactivation of gemcitabine due to deamination by cytidine deaminase or by dephosphorylation by cytoplasmic 5'-nucleotidases have also been reported as possible reasons for drug resistance.

hCNT1 is a high affinity transporter that transports a number of pyrimidine nucleoside-derived anticancer drugs. It uses sodium-mediated transport to move preformed nucleosides and nucleoside analog drugs, such as gemcitabine and capecitabine, against the concentration gradient and into the cell. It has been shown that hCNT1 is predominantly expressed in well-differentiated epithelial cells, such as intestinal villi and hepatocytes, and is regulated by differentiated promoting agents such as growth factors and cytokines. hCNT1 has also shown to be transiently expressed in human pancreatic cancer cells and mediate high affinity uptake of gemcitabine in a cell-cycle dependent manner. However, the relevance of hCNT1 expression and response to chemotherapy in various cancers is not clear and thus we will perform a more detailed study to identify its role in ovarian cancer.

The state of nucleoside transporters, especially hCNT1, in early and advance stage ovarian cancers and its role in governing gemcitabine sensitivity or resistance during the course of cancer progression and/or treatment is largely unknown. In a recent study by Farré et al. that examined numerous human ovarian cancers using a tissue array approach determined

heterogeneity in expression within the CNT and ENT families in ovarian cancer cells, depending on the histological type of ovarian cancer, demonstrating that it was possible that these transporters had differing roles in anticancer drug sensitivity in ovarian cancers[7]. It was also demonstrated that ovarian cancers that lack hCNT1 are associated with traditionally poor prognosis, like clear cell carcinoma, which also lacks hENT1 and hENT2, and is a highly aggressive tumor.[7] Further, the higher number of cases of negative expression of hCNT1, but not hENT1 or hENT2, that is observed in gynecological tumors may illustrate a correlation between a lack of hCNT1 expression and nucleoside drug resistance. In the study done by Jamieson et al, it was demonstrated that 1 μM of NBMPR, an inhibitor of hENT1 and hENT2, will limit the ability of araC, a nucleoside analog similar to gemcitabine, to enter exponentially growing ovarian cancer cells by ~90%.[8] While this suggests that hCNT1 is not a major player for gemcitabine uptake in human ovarian cancers, it is not clear whether hCNT1 expression is altered in ovarian cancer tissues or if this contributes to resistance towards nucleoside analogs. In this study, we demonstrate significant loss of hCNT1 expression in ovarian cancer cells as opposed to normal ovarian surface epithelial cells and that the machinery required to deliver hCNT1 to the cell surface is specifically altered in certain histological types of ovarian cancer.

CHAPTER TWO
MATERIALS AND METHODS

Cell Culture

The normal ovarian epithelial cells, IOSE 80 and IOSE 385, were received from the Canadian Ovarian Tissue Bank at passage 10. These were cultured in a 1:1 mixture of MCDB 105 medium and Medium 199 supplemented with 15% Fetal Bovine Serum (FBS). All experiments were conducted in cells that are in or between passage numbers 10 – 15. All ovarian cancer cell lines were obtained from the American Type Cell Culture (Manassas, Virginia) except the CaOV-3 and SKOV3 cells, which were received from Dr. Shelley Hooks at the University of Georgia. TOV-21G, TOV-112D and OV-90 were cultured in a 1:1 mixture of MCDB 105 medium and Medium 199 supplemented with 15% Fetal Bovine Serum (FBS). PA-1 was cultured in Eagles Essential Minimal Media with 10% FBS. ES-2 and SKOV-3 were cultured in McCoys 5A, 1X medium supplemented with 10% FBS. CaOV-3 and SKOV-3 were cultured in DMEM media supplemented with 10% FBS. The media for all cells were all purchased from MediaTech (Manassas, Virginia) and all media were supplemented with 100 units of penicillin/mL and 2µg streptomycin/mL in solution (Sigma-Aldrich, St. Louis, Missouri). The cells were kept at 37°C in a 5% CO_2 environment and all cell lines were subcultured at 48 – 72 hour intervals.

Retroviral Infection

The retroviral infection of various human ovarian cancer cell lines was performed as described previously (10). A retroviral vector, $LNCX_2$, containing human CNT1 fused to

hemagglutinin (HA) was constructed and designated $LNCX_2$ hCNT1 HA. This construct was transfected into human ovarian cancer cells using FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, Indiana). Control and recombinant retroviruses harboring hCNT1 cDNAs were produced in an amphotropic packaging cell line PA317. Cells were infected with recombinant viruses after 24 and 48 hours of plating. Transport experiments were conducted 24 hours after the second infection. For stable expression of hCNT1 HA, human ovarian cancer cell lines infected with $LNCX_2$ hCNT1 HA were selected in G418 (400 mg/mL active) for 2-3 weeks and individual clones were expanded and maintained in G418 (200 mg/mL).

Antibodies and Immunostaining

The primary antibodies used in the study we obtained from Santa Cruz Biotechnology, Santa Cruz, California. Immunolocalization of human CNT1 was performed as described previously (9). Briefly, the cells were grown to 80-90% confluence on glass cover slips in six well clusters. Cells were fixed with 2% paraformaldehyde (PFA) in 0.1M potassium phosphate buffer and then were blocked and permeabilized with 1% horse serum and 0.2% Trition-X100 for thirty minutes. This was then replaced with the primary antibody solution and incubated for one hour at room temperature. The cells were washed with phosphate buffer saline containing 0.02% Tween-20 three times for fifteen minutes each time. The cells were subsequently incubated in a secondary antibody solution, which contained antibodies conjugated with Alexa 488 or Alexa 592 (Invitrogen, Carlsbad, California), and cells were washed as before. The cover slips were mounted on glass slides using ProLong Gold antifade reagent with DAPI (Invitrogen, Carlsbad, California) as a nuclear fluorescent stain. The images were acquired with a Nikon

Eclipse Ti fluorescence microscope fitted with a 14-bit CCD camera (Nikon Instruments Inc., Melville, New York).

Western Blotting

The Western Blotting was done as described previously (9). Briefly, lysis of the cells was performed by incubating with lysis buffer (10mM Tris HCl, pH 8, 0.5% Nonidet P-40, 1mM EDTA, 2mM PMSF) on ice for ten minutes. Cells were homogenized by passing through a 21 G needle and followed by centrifugation at 10,000rpm for 3 minutes after which the supernatant was collected. The samples were heated at 100°C with loading dye and centrifuged at 1,500rpm for 30 seconds. The samples were separated on a 10% SDS gel and proteins were transferred onto a porous membrane (Pall Co., Pensacola, Florida) using a blotting apparatus. The membrane was washed, blocked, and incubated with primary antibodies followed by secondary HRP-conjugated antibodies (Bethyl Laboratories, Montgomery, Texas) where appropriate. Bound antibodies were detected using West Pico synthesized chemiluminescent reagent (Pierce Biotechnology, Rockford, Illinois). Images were then developed using a chemiluminescent machine.

Transport Studies

Cellular transport of ^{3}H-Thymadine and ^{3}H-Gemcitabinewere carried out as described previously (9). Briefly, ~5 x 10^{4} cells were plated on a 24-well plate in triplicate of wild type cells and in triplicate of hCNT1 viral cells. ^{3}H-thymidine (Moravek Radio Chemicals, Bria, California) transport studies were conducted in a sodium buffer containing 10 µM NBMPR (Sigma-Aldrich, St. Louis, Missouri), which inhibits hENT1 and hENT2 activity. The reaction

will be arrested and washed with a mixture of sodium free buffer and 10 mM uridine. To lyse the cells, 500 µL of 1N HCl was added followed by 500 µL 1N NaOH. Radioactivity was measured by an echoscintillation counter (Beckman Coulter, Fullerton, California). The results were examined graphically in Microsoft Excel (Microsoft, Redmond, Washington) and the change in level of transport between the wild type cells and the hCNT1 viral cells was determined.

Gemcitabine Toxicity Analysis

The cytotoxicity analysis was performed as described previously (11,12). Briefly, ovarian cancer cells were plated in a 96-well plate, where they were allowed to attach overnight. The samples were then treated to different serial concentrations of gemcitabine, and then the cells were incubated at 37°C for 72h. 50µL of 5mg/mL MTT was then added to the cells and they were incubated for two hours, after which 50µL DMSO dissolved the product of the reaction. The absorbance in each well was determined at 490 nm and 680nm by a Biorad Microplate Reader (BioRad, Hercules, California). The overall inhibition of each cell line was determined by performing the MTT assay in triplicate. The IC_{50} values for each cell line were determined from the cytotoxicity curves by using GraphPad Prism 5 (GraphPad Software, La Jolla, California).

CHAPTER THREE
RESULTS

Expression of Human Concentrative Nucleoside Transporter 1 is Decreased in Human Ovarian Cancer Tissues and Cell Lines.

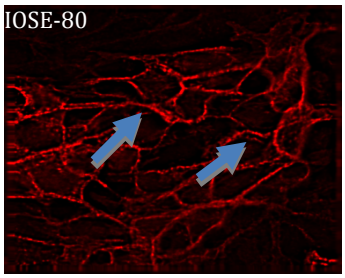

Figure 1: hCNT1 Expression in Normal Ovarian Cells

Previous experimentation has indicated that ovarian cancer cells have less active hCNT1 than normal ovarian cells. Immunostaining has indicated that none of the ovarian cancer cell lines that were tested have any active cell surface hCNT1 transporters, while the normal ovarian cell line, IOSE-80, had moderate cell surface expression of hCNT1 (See Figure 1). PA-1, OVCAR-3, and CaOV-3 showed intracellular hCNT1 expression, TOV-112D and SKOV-3 showed minimal cytoplasmic expression, and TOV-21G and OV-90 had nuclear expression of

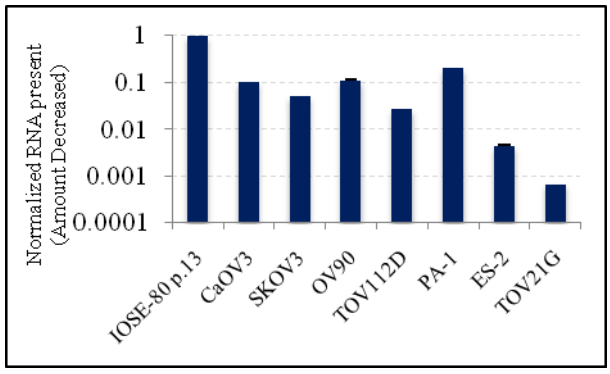

Figure 2: hCNT1 RNA in Normal and Cancerous Ovarian Cells

Table 1: hCNT1 Expression Determined by Immunostaining of Normal and Cancerous Ovarian Cells.

	Type of expression	IOSE-80	CaOV3	OV-90	SKOV3	TOV21G	PA-1	TOV112D
hCNT1	Cell surface	††	-	†	-	†	-	-
	Cytoplasmic	-	†	†	-	†	†	-

hCNT1 (See Table 1). RNA analysis has indicated that there is less RNA coding for hCNT1 in ovarian cancer cells compared to normal ovarian surface epithelial cells and the decrease is 5-1440 fold (See Figure 2).

Transport of ^{3}H-Gemcitabine is Greatly Reduced in Ovarian Cancer Cells

The transport of ^{3}H-gemcitabine by hCNT1 is much higher in the normal ovarian surface epithelial cells than in any of the ovarian cancer cells. The decrease in ^{3}H-gemcitabine transport ranges from a ~5 fold decrease by TOV21G to a ~35 fold decrease by TOV112D. Within these ovarian cancer cell lines, a significant loss of hCNT1 transport function occurs opposed to the normal ovarian surface epithelial cells (See Figure 3). The results are presented as the mean ± standard deviation from three experiments ($p<0.05$).

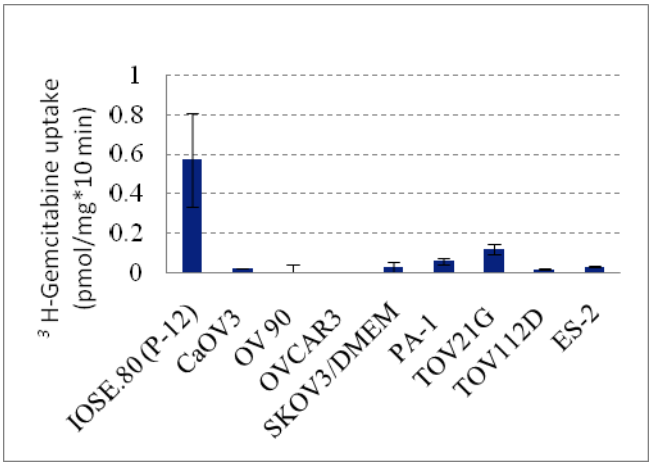

Figure 3: ^{3}H-gemcitabine Transport by Normal and Cancerous Ovarian Cells

Retroviral Expression of hCNT1

All cell lines were infected with $LNCX_2$ hCNT1 HA. Stable transduction was effective on the following cell lines was effective on most ovarian cancer cell lines. Transient transfection was performed on all cell lines to confirm data that was gathered using stable transduced $LNCX_2$ hCNT1 HA cells. After cells were selected with G418, they were frozen for later use.

Retroviral transport vs. Wild-Type Transport

Transport studies were performed on wild type cancer cells as well as cells infected with $LNCX_2$ hCNT1 HA. CaOV3, SKOV3 and TOV112D showed an increase in hCNT1 ^{3}H-gemcitabine transport from their wild-type counterparts after transfection with the $LNCX_2$ hCNT1 HA gene. ES-2, TOV21G, PA-1 and OV90 showed similar levels of transport in both types of cells (See Figure 4).

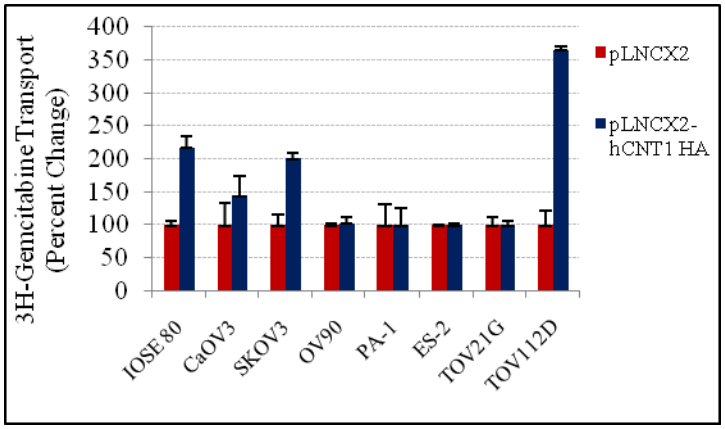

Figure 4: ^{3}H-gemcitabine Transport by Wild-type and Retrovirally Infected Ovarian Cancer Cells

Retroviral Expression vs. Wild-type Expression

After introduction of $LNCX_2$ hCNT1 HA into all ovarian cancer cell lines, immunostaining was performed to determine localization within the cells. $LNCX_2$ hCNT1 HA was localized at the cell surface in TOV112D, SKOV3, CaOV3, and OV90, while expression was

Table 2: hCNT1 Expression in Wild-type and Retrovirally Infected Cells.

Cell Line		Cytoplasmic Expression (hCNT1)	Cell Surface Expression (hCNT1)
Serous	CaOV3	†	† †
	SKOV3	†	† †
	OV90	†	† †
Clear Cell Carcinoma	ES-2	† †	-
	TOV21G	† †	-
Teratocarcinoma	PA-1	† †	-
Endometroid	TOV112D	-	† † †

cytoplasmic in TOV21G, ES-2, and PA-1.

Western Blotting

Western blotting was performed on both wild-type cells and transfected $LNCX_2$ hCNT1 HA cells. The $LNCX_2$ hCNT1 HA cells were probed with HA polyclonal and HA monoclonal antibodies and all showed expression, indicating that the $LNCX_2$ hCNT1 HA proteins were present in all transfected cell lines. Anti-hCNT1 antibodies were used on both sets of ovarian cancer cells, and it is apparent that there is a greater expression of hCNT1 in those cells that have been transfected with $LNCX_2$ hCNT1 HA than their wild-type counterparts. Multiple trials were performed to determine whether CNT1 was present in wild-type cells and increased in $LNCX_2$ hCNT1 HA transfected cells.

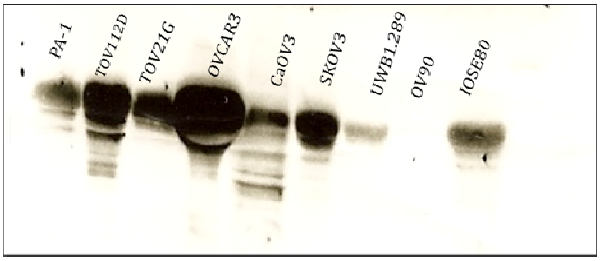

Figure 5: hCNT1 Expression in Wild-type Ovarian Cancer Cells

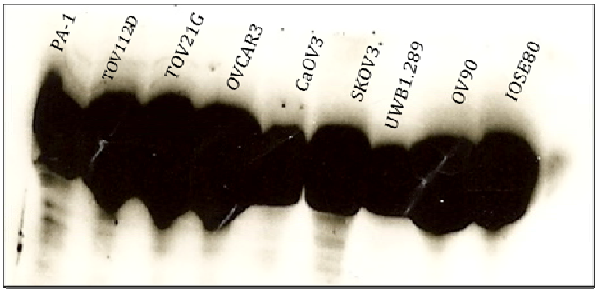

Figure 6: hCNT1-HA Expression in $LNCX_2$ hCNT1 HA Transfected Ovarian Cancer Cells

Cytotoxicity Analysis

The sensitivity of the transfected hCNT1 ovarian cancer cells to gemcitabine was determined to be cell subtype dependent. The Clear Cell Carcinomas (ES-2 and TOV21G) and teratocarcinoma (PA-1) had minimum change in their sensitivity to gemcitabine after transfection with $LNCX_2$ hCNT1 HA. Serous cells (OV90, CaOV3, and SKOV3) had a moderate

increase in sensitivity after transfection, with a ~25 fold increase in gemcitabine sensitivity. Endometroid carcinoma (TOV112D) had the highest overall increase in sensitivity, with a ~140 fold increase in gemcitabine sensitivity.

Table 3: IC_{50} values

Endometriod Carcinoma	TOV-112D	pLNCX2	3.8 ± 0.3 µM
		pLNCX2-hCNT1	26.8 ± 0.7nM
Serous Carcinoma	OV90	pLNCX2	200.5 ± 0.7nM
		pLNCX2-hCNT1	8.0 ± 0.8 nM
	CaOV3	pLNCX2	ND
		pLNCX2-hCNT1	ND
	SKOV3	pLNCX2	ND
		pLNCX2-hCNT1	ND
Teratocarcinoma	PA-1	pLNCX2	5.6 ± 0.01 nM
		pLNCX2-hCNT1	5.2 ± 0.6 nM
Clear Cell Carcinoma	ES-2	pLNCX2	4.5 ± 0.04 µM
		pLNCX2-hCNT1	5.1 ± 0.5 µM
	TOV-21G	pLNCX2	4.8 ± 0.4 nM
		pLNCX2-hCNT1	12.0 ± 2.0 nM

CHAPTER FOUR
DISCUSSION

Our research has focused exclusively on hCNT1 transport in ovarian cancer cell lines. This transporter has been implicated in sensitizing solid tumors (pancreatic and breast) towards gemcitabine, and it has been approved for use in late stage or highly resistant ovarian cancer, but its exact role in increasing gemcitabine efficacy is unknown. Initially, we used RNA analysis to compare the hCNT1 RNA in normal ovarian surface epithelial cells and ovarian cancer cells. This indicates a decrease (in 5-1440 fold) in hCNT1 RNA within the ovarian cancer cell lines. Immunostaining was also used to determine the location and activity of hCNT1 transporters in wild-type ovarian cancer cell lines. We determined that hCNT1 surface expression is limited to normal surface ovarian epithelial cells, and is not present in any ovarian cancer cell line, which may indicate a loss of functionality in these cell lines. Transport studies also indicate a loss of functionality, with a 3-10 fold decrease in ^{3}H-gemcitabine transport in all ovarian cancer cell lines compared to normal ovarian surface epithelial cells. All of this determines that there is a decrease in hCNT1 expression within the ovarian cancer cell lines tested, and while there may be indications that the hCNT1 proteins are present, it is obvious that the ovarian cancer cell lines are unable to utilize them for any type of extracellular transport.

The next step in our research was to virally infect all cell lines with $LNCX_2$ hCNT1 HA, a gene which codes for the hCNT1 transporter, plus an hemoagglutinin (HA) tag which we could use to confirm its presence in Western Blots and immunostaining. After transfection and selection, we were able to confirm by immunostaining the presence of active hCNT1 transporters

in multiple cell lines were they were not present before; these cell lines were SKOV3, TOV112D, CaOV3, and OV90. In transport studies, SKOV3, CaOV3, and TOV112D showed an increase in ^{3}H-gemcitabine uptake of hCNT1 in cells with $LNCX_2$ hCNT1 HA. PA-1, TOV-21G, ES-2, and OV90 showed no change in hCNT1 activity between wild-type cells and $LNCX_2$ hCNT1 HA transfected cells.

In the cytotoxic assay, we examined the cell's direct response to different levels of gemcitabine. The response was cell-subtype correlated, which was initially unexpected to us. The endometroid carcinoma, TOV112D had the highest change in sensitivity to gemcitabine, with an IC_{50} value of 26.8 ± 0.7 nM after $LNCX_2$ hCNT1 HA infection, which was a ~140 fold decrease from its original IC_{50} value. This is not surprising, as this ovarian cancer subtype is not known for its chemotherapeutic resistance. The serous carcinomas (OV90, CaOV3, SKOV3) all showed a moderate increase in sensitivity to gemcitabine (~25 fold decrease). The ovarian cancer cells with the least sensitivity to gemcitabine, even after infection with $LNCX_2$ hCNT1 HA, were the teratocarcinoma (PA-1) and the clear cell carcinomas (ES-2 and TOV21G). These cell lines showed almost no change in sensitivity to gemcitabine after $LNCX_2$ hCNT1 HA exposure. In immunostaining, clear cell carcinomas showed a misdirection of $LNCX_2$ hCNT1 HA into the Golgi, which may have resulted in minimal surface expression comparable to the level in the wild-type clear cell carcinoma cells. The PA-1 cells, teratocarcinoma cells, showed no increase in gemcitabine sensitivity either, and showed misdirection of hCNT1 HA to intracellular vesicles. This will require further research to determine the obstacles present in clear cell carcinoma and teratocarcinoma cells blocking the expression and/or uptake of $LNCX_2$ hCNT1 HA.

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