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Testing the advantage of a cancer-associated glutamine transporter in liver cells

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NORTHERN ILLINOIS UNIVERSITY

TESTING THE ADVANTAGE OF A CANCER-ASSOCIATED GLUTAMINE TRANSPORTER IN LIVER CELLS

A thesis submitted to the University Honors Program in partial fulfillment of the requirements of the Baccalaureate Degree with upper division Honors

Department of Biological Sciences

By

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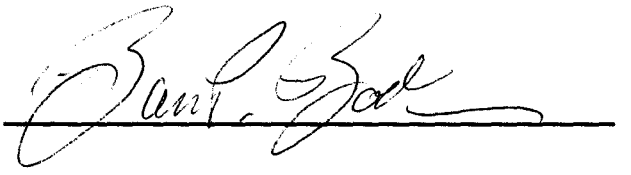
Faculty Approval Signature 

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ABSTRACT: Neutral amino acid transporter ASCT2 is selectively over-expressed in cancerous liver cells. The purpose project was to test why this amino acid transporter was being expressed and to inquire the implicit advantage ASCT2 gives a cancer cell. This topic is of significance due to its relevance to cancer biology and of the prospect of designing cancer drug therapies that can exploit the ASCT2 transporter under inquiry. Growth study curves using varying concentrations of glutamine and cysteine with cell lines RLE human, rat, mouse and control empty vector were conducted to illustrate possible advantage in survival and growth. A Western blot assay was also performed to confirm the amplified expression of the ASCT2 in the cell lines used for the growth studies. The growth study results implicated no relevant growth advantage for the RLE cell lines that over-express ASCT2 compared to the control. The results may be explained by the western blot assay results, in accordance, that showed no explicit over expression of the ASCT2 protein in the rle cell lines in comparison to the empty vector cell line used for the growth study.

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1. Introduction

The purpose of my senior honors capstone project was to test the teleological question of why a specific amino acid transporter known as ASCT2 is predictably expressed and activated in cancerous liver cells. The hypothesis that was drawn from past experimental studies and background knowledge suggested that due to the higher affinity of ASCT2 for one of its neutral amino acid substrate glutamine in relative comparison to other normal cell transporters, ASCT2 expression will allow cells to grow better under conditions of low glutamine concentrations, as might occur inside a tumor microenvironment. Experimentally inquiring the implicit advantage ASCT2 gives a cancer cell is a topic of interest and importance due to its relevance to cancer biology and also of the future prospect of designing cancer drug therapies that is specifically targeted to exploit the ASCT2 transporter under inquiry. The lab research is intended to illustrate the correlation between ASCT2 transporter expression and the possible advantage this transporter gives cancer cells in surviving low concentrations of glutamine or cysteine and the growth of cancer.

2. Background

ASCT2 is a neutral amino acid transporter that is not relevantly present or active in normal liver tissue cells. However when liver cells turn cancerous, the expression and activity of the ASCT2 transporters is considerably up-regulated. ASCT2 is a neutral amino acid transporter that utilizes the transmembrane Na^+ electrochemical gradient maintained by Na+/K+ ATPases to transport neutral amino acids into the cell including glutamine, cysteine, alanine, serine and threonine.

3. Project Design

To experimentally establish the survival and growth advantage ASCT2 transporter impose on the hepatocarcinoma cells, three liver cell lines expressing either rat, mouse or human ASCT2 along with a control empty vector cell was used to conduct multiple growth study curves under conditions of decreasing of glutamine concentration increments in media. The empty vector contained the vector used to express ASCT2 in the other three RLE cell lines including the Hygromycin resistant gene used to select for ASCT2 expression. After the cells were exposed to the variant glutamine gradient concentrations, a cell count was obtained to analyze the effect of ASCT2 had on the cells in low glutamine concentrations. Along with the growth curve studies, a western blot analysis was done on RLE mouse, RLE human and the empty vector cell lines to assess continued amplified ASCT2 expression.

Materials: Cell lines: RLE (Rat Liver Epithelial) Empty Vector

RLE Human ASCT2

RLE Mouse ASCT2

RLE Rat ASCT2

Dulbecco's Modified Eagle Medium (DMEM)

Fetal Bovine Serum (FBS)

L - Glutamine and L- Cysteine

Hygromycin

Phosphate Buffered Saline (PBS)

Trypsin (HyQTase)

Antibiotic/Antimycotic

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

Western Blot:

4x Sample loading buffer (SLB)

DTT

Lysis buffer

1x tank transfer buffer

Methanol

BlockHen

Purified Chicken primary antibody

Tween 20

Nitrocellulose blot membrane

Goat secondary antibody

LumiGLO

Hydrogen Peroxide

Nanopure water

Equipment : Incubator, Sterile hood, Western blot apparatus, blot imager and Nanodrop

Methodology

The four cell lines were thawed and grown in 25 ml flask in DMEM media containing FBS and Hygromycin. After optimal confluence was reached, the cells were aspirated of the original media, washed with PBS, and added trypsin in order for re-suspension into new media. A diluted cell containing media with relatively equal cell density was plated

into 48 well plates for the first study. Once the cells were grown over 24 hours, they were re-suspended in DMEM media containing zero glutamine concentration using the previously described methods. Then 12 different glutamine concentrations containing media were added to four consecutive wells plated for each four cell lines, RLE Empty Vector, RLE Human, RLE Mouse and RLE Rat and allowed to grow at 37^0C in incubators. The glutamine concentrations ranged from .050 M, 0.1 M, 0.2 M, 0.3 M, 0.4 M, 0.6 M, 0.8 M, 1.0 M, 1.5 M, and 2.0 M. After cells were grown in their respective glutamine concentration over two days, the cells were again re-suspended in media as described above. For our first glutamine growth curve study a dissolved MTT solution was added to the cells and given adequate time for uptake.

A second glutamine growth study using the same concentrations was later performed for only the RLE human and the control empty vector cell lines in 12 well plates to confirm the results of the first MTT assay results due to discrepancy in data from the first study.

Thirdly, a cysteine growth study using EV and human RLE lines identical to the second glutamine growth study was also carried out. Direct cell count method was used to collect the data from these two 12 well plates similarly. The concentrations of 0, 2, 5, 10, 20, 30, 40, 50, 75, 100, 150 and 200 μM cysteine were used for this study.

For the fourth cysteine growth study using EV and human RLE lines was later done to confirm the previous results. Direct cell count method was used to collect the data from these two 12 well plates similarly. The concentrations of 0, 2, 5, 10, 20, 30, 40, 50, 60, 70, 80 and 100 micro-molar cysteine were used for this study.

Western Blot: After the growth studies were concluded, a western blot assay was done on RLE mouse, human and the empty vector control to confirm the amplified expression of the ASCT2 in the RLE cell lines. Proteins isolated from all three of the cell lines were loaded onto a western blot gel apparatus mixed with SLB, DTT and lysis buffer along with a biotin and a three color protein molecular weight ladder for over an hour. The gel had biotin marker on the left, followed by empty vector, mouse, human and then the three colors. Then gel was then placed onto a tank transfer apparatus and blotted onto the nitrocellulose membrane. The blot paper was dried over night and a diluted BlockHen solution in PBS (5ml BlockHen in 20ml PBS) was used to incubate the membrane blot for an hour at room temperature with gentle rocking. Once done, the blot was washed three times for five minutes with 25ml of PBS. A group of purified IgY factions were read on the Nanodrop for the highest protein concentration. Based on the Nanodrop readings, 40µl of IgY with the highest protein concentration was added to 500ml of PBS and .5ml of Tween 20 solution. The blot membrane was then incubated in the primary antibody solution at 4°C overnight on a rocking platform. The third day, the blot was washed again four times using PBST solution. Then secondary goat antibody solution was added and incubated for an hour with gentle rocking. After an hour, the blot was washed again four timed with PBST. To the blot, .5ml of LumiGLO, .5ml peroxide reagent and 9 MLnanopure water was added for 1 minute at room temperature with gentle agitation and imaged.

Data Collection:

The MTT cell count assay uses the MMT compound solution that is originally a clear yellow solution that gets taken up by living cells, which later gets reduced and converted

to a chemogenic metabolite within the cell mitochondria to produces a purple insoluble dye that later can be measured as an absorbance using a spectrophotometer. The absorbance values correspond to the number of viable cells in the sample.

The second glutamine confirmatory growth curve study we carried out used direct cell count method were re-suspended cells were directly counted from a hemocytometer grid. The valued corresponding to the four grids on the hemocytometer were counted individually and averaged to get the cell count density per cell for each concentration.

The third and fourth cysteine growth study using EV and human RLE lines also used the direct cell count method to collect the data from these two 12 well plates similarly.

Western Blot: Then western blot was imaged using a chemiluminescence detector with five minute exposure.

4. Results

Note: The MTT assay spectrophotometer absorbance reading for the first glutamine study gave comparatively high values in the first two columns due to possible contamination or spectrophotometer reading error. These values were not factored into calculate the average cell density for the corresponding plate wells. The absorbance data for RLE rat cell line pertaining to the final two glutamine concentrations were also not recorded by the spectrophotometer.

The cell counts for the four grids on the hemocytometer were averaged and the standard deviation (SD) was also calculated.

Study One Glutamine Data using MTT Assay Method for EV, Human, Rat, and Mouse RLE

RLE Human MTT Assay Results

[GLN]	Count 1	Count 2	Count 3	Count 4	AVG	SD
0	x	x	0.077	0.075	0.075	0.001
0.025	0.08	0.08	0.075	0.078	0.076	0.002
0.05	x	x	0.096	0.101	0.099	0.004
0.1	0.101	0.104	0.104	0.096	0.101	0.004
0.2	x	x	0.124	0.116	0.120	0.006
0.3	0.121	0.124	0.135	0.119	0.125	0.007
0.4	x	x	0.179	0.167	0.173	0.008
0.6	0.16	0.167	0.158	0.161	0.162	0.004
0.8	x	x	0.174	0.188	0.181	0.010
1	0.188	0.19	0.173	0.252	0.201	0.035
1.5	x	x	0.208	0.202	0.205	0.004
2	0.187	0.195	0.156	0.207	0.186	0.022

RLE Mouse MTT Assay Results

[GLN]	Count 1	Count 2	Count 3	Count 4	AVG	SD
0	x	x	0.072	0.078	0.075	0.000
0.025	0.077	0.074	0.072	0.081	0.065	0.004
0.05	x	x	0.087	0.08	0.084	0.005
0.1	0.09	0.1	0.093	0.107	0.098	0.008
0.2	x	x	0.113	0.112	0.113	0.001
0.3	0.115	0.114	0.121	0.108	0.115	0.005
0.4	x	x	0.173	0.158	0.166	0.011
0.6	0.147	0.154	0.137	0.136	0.144	0.009
0.8	x	x	0.162	0.151	0.157	0.008
1	0.123	0.132	0.12	0.11	0.121	0.009
1.5	x	x	0.156	0.136	0.146	0.014
2	0.14	0.133	0.148	0.122	0.136	0.011

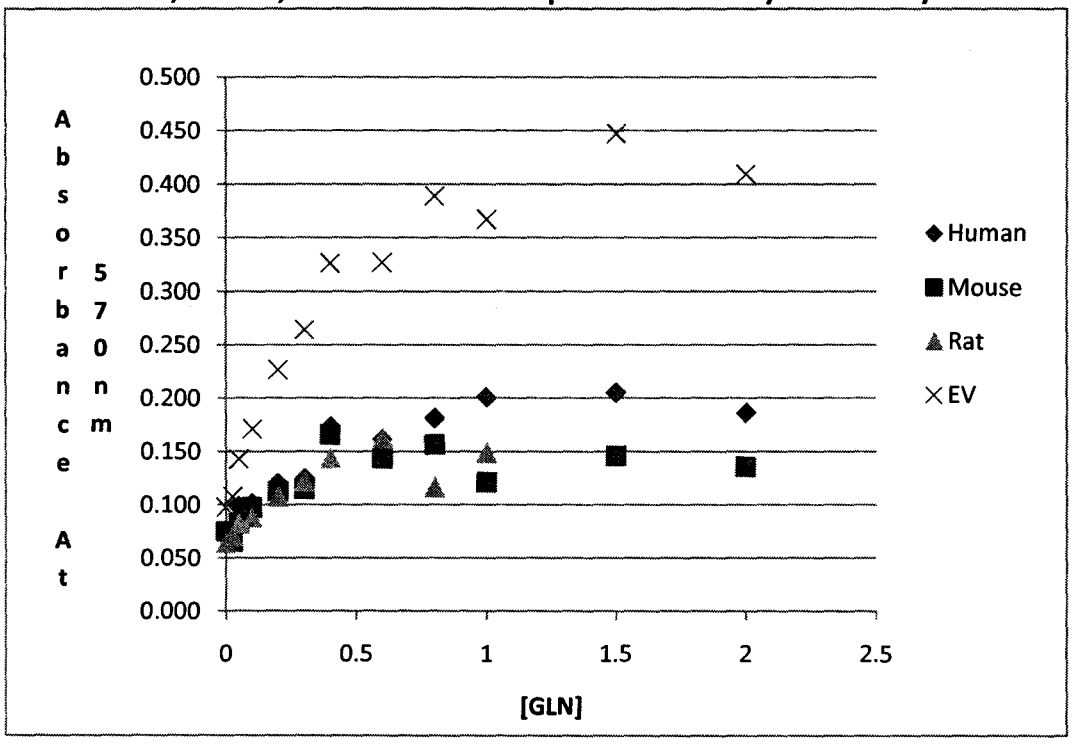
RLE Rat MTT Assay Results

[GLN]	Count 1	Count 2	Count 3	Count 4	AVG	SD
0	X	X	0.063	0.065	0.064	0.001
0.025	0.075	0.071	0.071	0.064	0.070	0.005
0.05	X	X	0.082	0.083	0.083	0.001
0.1	0.072	0.088	0.096	0.096	0.088	0.011
0.2	X	X	0.113	0.103	0.108	0.007
0.3	0.128	0.117	0.124	0.119	0.122	0.005
0.4	X	X	0.124	0.163	0.144	0.028
0.6	0.183	0.138	0.175	0.154	0.163	0.020
0.8	X	X	0.143	0.128	0.116	0.011
1	0.164	0.131	0.152	0.147	0.149	0.000

RLE Empty Vector MTT Assay Results

[GLN]	Count 1	Count 2	Count 3	Count 4	AVG	SD
0	X	X	0.1	0.096	0.098	0.003
0.025	0.116	0.111	0.107	0.096	0.108	0.009
0.05	X	X	0.142	0.144	0.143	0.001
0.1	0.18	0.18	0.177	0.148	0.171	0.016
0.2	X	X	0.22	0.233	0.227	0.009
0.3	0.288	0.255	0.271	0.243	0.264	0.020
0.4	X	X	0.342	0.311	0.327	0.022
0.6	0.337	0.341	0.319	0.311	0.327	0.014
0.8	X	X	0.384	0.394	0.389	0.007
1	0.377	0.377	0.389	0.327	0.368	0.028
1.5	X	X	0.461	0.434	0.448	0.019
2	0.429	0.425	0.399	0.385	0.410	0.021

Human, Mouse, Rat and EV RLE Graph for MTT Assay from Study One



Study Two Glutamine Data using Direct Cell Count Method for EV and Human RLE

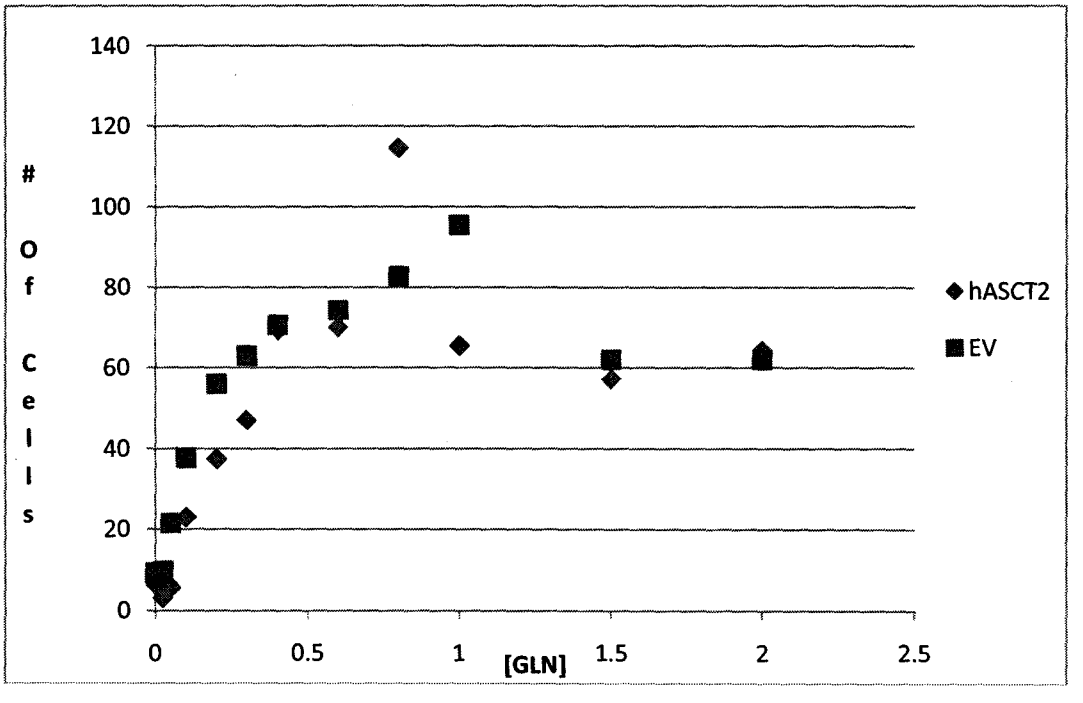
RLE Human Direct Cell Count Results

[GLN]	Count 1	Count 2	Count 3	Count 4	Avg	SD
0	9	6	6	5	7	2
0.025	5	3	3	2	3	1
0.05	7	5	3	8	6	2
0.1	24	31	14	23	23	7
0.2	47	44	38	21	38	12
0.3	37	59	49	43	47	9
0.4		80	71	51	67	15
0.6	64	82	71	63	70	9
0.8	110	128	90	130	115	19
1	51	71	98	42	66	25
1.5	47	70	55	57	57	10
2	66	74	45	72	64	13

RLE Empty Vector Direct Cell Count Results

[GLN]	Count 1	Count 2	Count 3	Count 4	Avg	SD
0	14	9	9	5	9	4
0.025	8	15	10	6	10	4
0.05	22	34	17	13	22	9
0.1	43	48	28	32	38	9
0.2	62	59	45	58	56	8
0.3	81	67	61	43	63	16
0.4	59	70	72	81	71	9
0.6	80	68	69	80	74	7
0.8	88	79	75	89	83	7
1	101	100	89	92	96	6
1.5	64	49	69	66	62	9
2	67	72	45	64	62	12

Human RLE and Empty Vector RLE Direct Cell Count Graph from Study Two



Study Three Cysteine Data using Direct Cell Count Method for EV and Human RLE

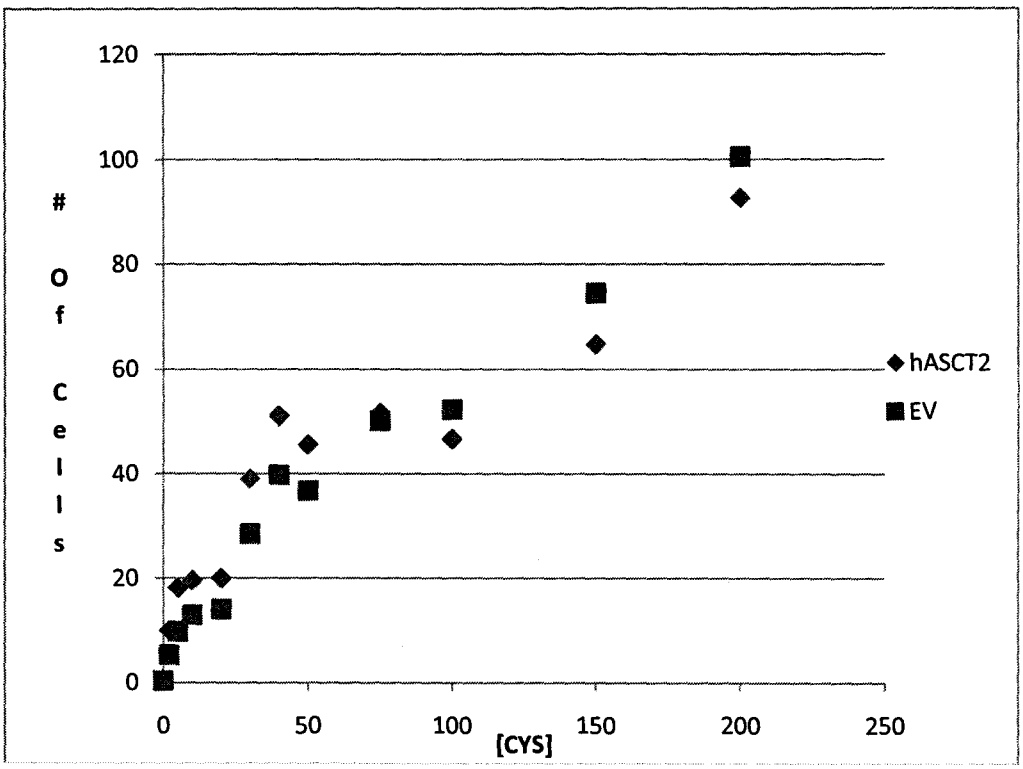
RLE Human Direct Cell Count Results

[CYS]	Count 1	Count 2	Count 3	Count 4	Avg	SD
0	2	1	1	1	1	1
2	29	25	21	20	24	4
5	38	37	48	49	43	6
10	28	51	38	40	39	9
20	39	37	35	40	38	2
30	54	88	81	60	71	16
40	110	107	52	71	85	28
50	59	88	81	60	72	15
75	29	43	78	74	56	24
100	26	44	20	29	30	10
150	48	35	28	31	36	9
200	72	66	80	59	69	9

RLE Empty Vector Direct Cell Count Results

[CYS]	Count 1	Count 2	Count 3	Count 4	Avg	SD
0	2	1	3	3	2	1
2	28	23	8	15	19	9
5	63	70	39	50	56	14
10	46	48	27	39	40	9
20	85	89	98	54	82	19
30	31	48	27	29	34	10
40	140	131	119	123	128	9
50	148	111	116	104	120	19
75	125	104	105	107	110	10
100	119	103	109	128	115	11
150	127	105	138	91	115	21
200	124	117	130	151	131	15

Human RLE and Empty Vector RLE Direct Cell Count Graph from Study Three



Study Four Cysteine Data using Direct Cell Count Method for EV and Human RLE

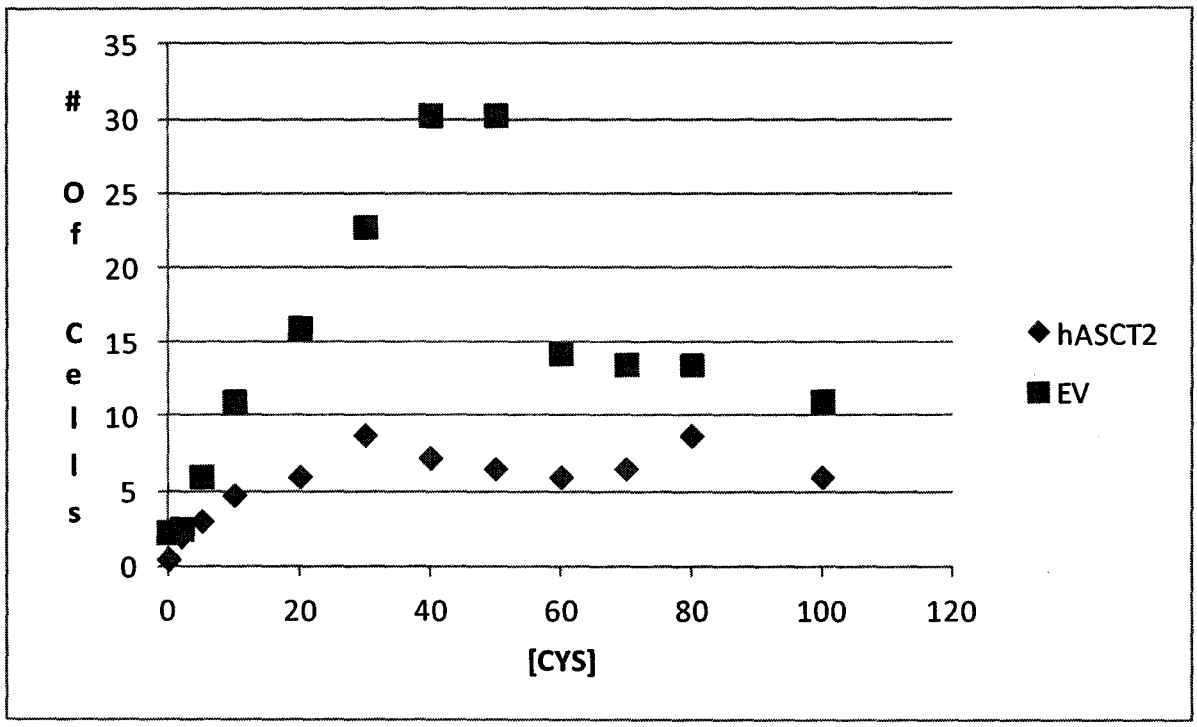
RLE Human Direct Cell Count Results

[CYS]	Count 1	Count 2	Count 3	Count 4	Avg	SD
0	1	1	0	0	1	1
2	2	4	1	1	2	1
5	4	1	4	3	3	1
10	4	10	2	3	5	4
20	5	7	6	6	6	1
30	9	12	7	7	9	2
40	4	3	10	12	7	4
50	11	4	2	9	7	4
60	4	9	5	6	6	2
70	10	5	4	7	7	3
80	9	11	7	8	9	2
100	7	7	4	6	6	1

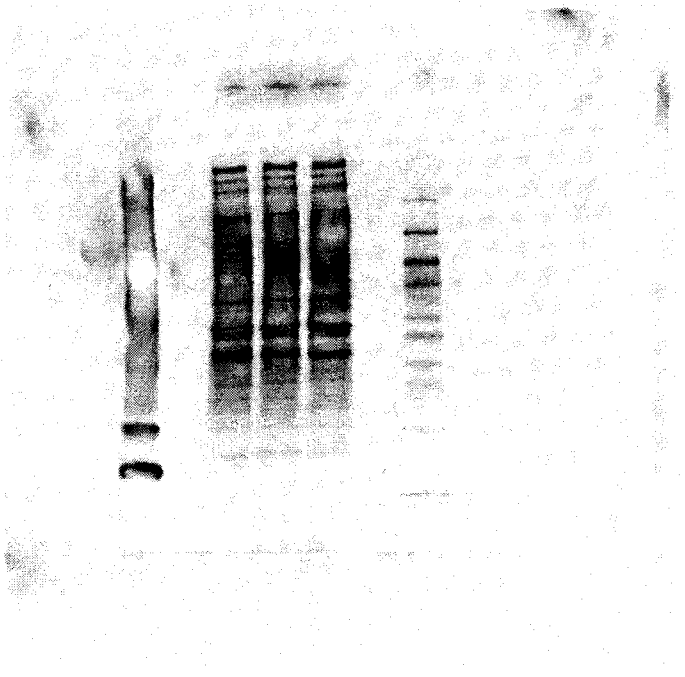
RLE Empty Vector Direct Cell Count Results

[CYS]	Count 1	Count 2	Count 3	Count 4	Avg	SD
0	3	1	1	4	2	2
2	3	3	2	2	3	1
5	8	9	5	2	6	3
10	9	16	11	8	11	4
20	12	15	23	14	16	5
30	27	24	26	14	23	6
40	34	33	29	25	30	4
50	24	34	37	26	30	6
60	13	10	13	21	14	5
70	11	17	13	13	14	3
80	17	16	9	12	14	4
100	5	12	9	18	11	5

Human RLE and Empty Vector RLE Direct Cell Count Graph from Study Four



Western Blot Image



5. Data Analysis and Interpretation

The data for study one and study two using gradients of glutamine concentrations show that the ASCT2 expressing cell lines did not exhibit significant growth advantage compared to the control empty vector cell line. This can be theorized due to the fact that increase expression and activity of ASCT2 transporter only enables the cell to uptake the glutamine but the metabolic limitations in-build to the cells prevent the increased metabolite transport to translate into increased cell growth.

The third growth study done for cysteine however showed comparative growth advantage for the RLE cell line expressing ASCT2 compared with the control empty vector cell line. This could be due to the fact low concentrations of cysteine in the extracellular space could trigger up-regulated cysteine transport into the cell via ASCT2 where the cysteine along with glutamine can be later used to manufacture essential

compounds such as glutathione. Glutathione production in cancer cells protects the cell from oxidative stress. However, the fourth and final cysteine growth study showed the empty vector cell line having a higher growth advantage compared to the human ASCT2 cell line that contradicted the rationalization above. Thus, results were equivocal.

Western blot: The western blot preliminary data shows a triplet band at around 55kDa which is possibly the ASCT2 band along with multiple bands above and below. There seems to be no band intensity difference among the three cell lines used suggesting that the RLE cell lines comparatively do not have amplified expression than the control empty vector cell line. The multiple bands that appeared on the western blot could be due to error in antibody purification method, incorrect western wash or incubation conditions.

6. Conclusions

Based on the glutamine growth curve data, we can deduce that the expression of ASCT2 is not the growth-limiting factor for growth in human ASCT2 expressing cell line. The intrinsic metabolic capacity of the liver cells only allows a portion of the glutamine being transported by the ASCT2 transporter to be utilized for growth and proliferation. The fact that cells are not metabolically equipped to use the excess glutamine that's up-taken explains the lack of growth response at varying glutamine concentrations.

The first cysteine growth curve data implies that expression of ASCT2 does in-fact give the human RLE cell line a growth advantage under low cysteine concentrations. But the second cysteine growth study which was done focusing on even lower

concentrations disproved the previous results and showed that the empty vector had a relative growth advantage compared to the human ASCT2 cell line.

The conclusive western blot assay showed that the RLE cell lines over-expressing ASCT2 did not have amplified ASCT2 protein expression compared to the control empty vector cell line. Further glutamine transport assays needs to be performed to confirm this premise. Refining the westerns protocol by re-purify antibodies or finding better washing or incubation conditions can help confirm the preliminary results. The lack of over expression in the RLE cell lined used in the study could be due to the extended period of time since they were created and last confirmed. The cell lines could have possibly maintained the Hygromycin resistance but lost ASCT2 expression over time. Further kinetic and western assay needs to be performed to conclude this assumption.

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