HnRNPA1 and Its Effect in the Expression of ABCC (ABCC4 and ABCC6) Transporter in Glioma Cell Lines

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Key words: hnRNPA1, ABCC family genes

Abstract:

Glioblastoma multiforme (GBM) is classified as WHO grade IV Astrocytoma & is the most common highly aggressive form of primary brain tumor. Garde IV tumor are highly recurrent even after treatment, with patient survival rate is less than two years from the time of diagnosis. This might be due to overexpression of one of the factors such as ATP-binding cassette transporters (ABC transporters) responsible for drug resistance. ABCC transporter family a member of ABC transporter was found to mostly responsible for multi drug resistance (MDR) in cancer cells. On the other hand, heterogeneous nuclear ribonucleoprotein (hnRNP’s) an alternative splicing factor play different role in various cellular process such as nucleic acid metabolism, transcription and translation regulation, among them hnRNPA1 is best studied and its aberrant deregulation favor development of cancer. This study was focused on to study the function of hnRNPA1 in the expression analysis of ABCC transporter (responsible for MDR) in glioma cell
The expression of ABCC transporter (ABCC4 and ABCC6) gene was examined in two glioma cell line i.e. U87MG and T98G in normal and knockdown two alternative variants of hnRNPA1 by Quantitative Realtime PCR and Reverse Transcription PCR. We found that ABCC4 was significantly overexpressed in hnRNPA1 Variant 2 knockdown cells (si hnRNPA1 V2) in U87 (3-fold) and in T98G (18.34-fold), While hnRNPA1 Variant 1 knockdown cells (hnRNPA1 V1i) does not shows any significant effect. Further, the expression of ABCC6 was decreased in both hnRNPA1 V1i (0.40-fold) and hnRNPA1 V2i (0.48-fold) in U87. Reverse transcription-based result was complemented with normal PCR based detection strategy after running in agarose gel for U87MG and T98G glioma cell line. Finally, this result indicates that hnRNPA1 an alternative splicing factor regulate the expression of ABCC4 and ABCC6 transporter which are responsible for multiple drug resistance in cancer. This information will help in future for the development of an alternative method for the treatment of drug resistance cases in brain tumor and other tumors by targeting hnRNPA1 splicing factor.
Introduction:

Glioblastoma Multiforme (GBM) is the most common and highly aggressive form of primary brain tumor [1]. It develops from glial cell, supporting cells of neurons [2]. Based on the tumor location and aggressiveness, tumors are graded from grade I to IV and GBM is often classified as WHO Grade IV Astrocytoma [3]. They are highly aggressive and tend to spread into nearby brain tissues. The treatment strategies include surgically resection of tumor mass followed by radiotherapy and chemotherapy (usually temozolomide) [4]. Over the past few decades, disease has been highly researched but did not result into clinical outcome of patient [5]. GBM is highly recurrent even after the treatment and results into poor prognosis. The survival rate is less than two years from the time of diagnosis [6].

ATP-binding cassette transporters (ABC transporters) are ATP dependent pumps that transport (export and import) various substrates across the membranes [7]. In human, there are 48 human ABC transporter genes classified into 7 subfamilies. They are ABCA (12 members), ABCB (11 members), ABCC (12 members), ABCD (4 members), ABCE (1 member), ABCF (3 members), and ABCG (5 members) [8]. Among them three subfamilies, ABCB, ABCC and ABCG, play a key function at the BBB (Blood Brain Barrier), protecting the brain against the entry of xenobiotics and decreasing the accumulation of metabolic products [9]. ABCC family members are often attributed to multidrug resistance (MDR) in cancer cells [10]. Many of these ABCC transporters are overexpressed in various cancers and confer chemoresistance by efflux transport of anticancer drugs through cotransport with glutathione or glutathione-drug or glucuronide-drug conjugates [11], [12].

Alternative splicing is a post-transcriptional process which produces alternative mRNA transcripts [13]. These splice variants encode for protein isoforms which could have distinct and even antagonistic properties [14]. This process of alternative splicing is carried out by spliceosome. The spliceosome consists of five small nuclear ribonucleoproteins (snRNPs) - U1, U2, U4, U5 and U6
RNA-binding proteins (RBPs) such as heterogeneous nuclear ribonucleoprotein (hnRNP), arginine–serine-rich (SR) and RNA-binding motif (RBM) proteins are splice factors that recognize the regulatory elements in introns and exons and promotes or suppress the recognition of nearby splice sites by splice machinery [16]. In human cancers, alternative splicing is deregulated in favor of cancers [17]. Various evidences have suggested the role of aberrant alternative splicing in development and progression of cancer. In fact, aberrant alternative splicing is another hallmark of cancer [18]. Heterogeneous nuclear ribonucleoproteins (hnRNPs) are RNA binding proteins (RBPs) with diverse role in various cellular processes such as nucleic acid metabolism, transcription and translation regulation [19]. Among various hnRNPs, hnRNPA1 is the best studied hnRNP members in human [20]. Overexpression of hnRNPA1 promotes tumor invasion in gastric cancer [21] and also promotes Beclin-1 (also known as autophagy-related protein 6) expression in colorectal cancer [22]. It also regulates TIMP1 intron 3 retention in colorectal cancer [23], thus an important marker for colorectal cancer progression. HnRNPA1 also promotes hepatocellular carcinoma invasion [24]. It is strongly expressed in in ERG-negative prostate cancer and lung cancer [25], [26]. HnRNPA1 along with hnRNPA2 and PTB, preferentially include exon 10 (PKM2) by binding repressively to exon 9 flanking sequences [27]. This shift from PKM1 to PKM2 promotes aerobic glycolysis and positively support to tumor formation. It is also reported to be overexpressed in human glioma [28], [29].

The ATP-binding cassette (ABC) transporters are a family of transporter proteins that are responsible for drug resistance and chemoresistance in cancers and other diseases [10]. Overexpression of ABC transporters such as P-glycoprotein (ABCB transporters), MRPs (ABCC transporters) and Breast Cancer Resistance Protein (BCRP, ABCG2) are known to be responsible for the most of Multidrug resistance (MDR) phenotypes [11], [12]. On the other hand, Heterogeneous nuclear ribonucleoproteins (hnRNPs) are RNA binding proteins (RBPs) that play an important role in various cellular processes such as nucleic acid metabolism, transcription and
translation regulation [19]. HnRNPA1 (member of hnRNP family) is the most abundant and ubiquitously expressed member of this protein family and has been shown to be involved in multiple molecular events driving malignant transformation [30]. The expression of ABCC transporters and the role of hnRNPA1 in its expression was not properly investigated in the human glioma. Therefore, in the current study we investigated the expression of ABCC transporters (ABCC2, ABCC3, ABCC4, ABCC5 and ABCC6) and the role of hnRNPA1 alternative variants (hnRNPA1 V1: Accession:NP_002127.1,GI: 4504445; hnRNPA1 V2: Accession: NP_112420.1,GI: 14043070) which are relatively expressed in Glioma cells are confirmed (unpublished work). Functional redundancy or distinct in nature were demonstrated in context to ABCC 4 & ABCC6.

Material & Methods:

Primer Designing:

Common parameters of primers in qRT-PCR were taken into consideration. Primers were designed using Primer-Blast and mRNA sequences were retrieved from NCBI gene database [69]. The primer only showing predicted amplicons on intended target i.e. having high specificity was chosen. The primers used are listed in Table 1. For ABCC4 the forward and reverse primer were made from exon 3 and exon 4 respectively (Fig 1). While for ABCC6, the forward primer is from the junction of exon 22 and 23 while the reverse is from exon 24 (Fig 2).

Cell culture Material:

U87, U373, T98G, A172 & LN229 cells (Generous support from Prof. Subrata Sinha, N.B.R.C, Manesar, Gurgaon (India) were propagated in Dulbecco’s modified Eagle’s medium (DMEM) (PAN Biotech) composition: 10% fetal bovine serum (PAN Biotech), 100 units/ml penicillin-streptomycin (PAN Biotech). Cells were grown under 37°C and humidified chamber under 5% CO2.

si RNA transfection:
U87 and T98G cells were transfected next day after plating, with specific hnRNPA1(V1) [10nM] and hnRNPA1 (V2) (10nM), siRNA and scrambled siRNA (Santa Cruz Biotechnology, Inc.) as per protocol. Protein lysates were prepared after 72 hrs of transfection in cell lysis buffer (Cell Signaling Technology) containing protease inhibitor (Abcam) and phosphatase inhibitor (Santa Cruz Biotechnology, Inc.). In all experiment siRNA mediated knock down efficiency was achieved ≥ 50% to 60 % using transfection reagent from Mirus Company. All experiment was performed at least three times.

**Semiquantitative PCR & Quantitative PCR (Polymerase Chain Reaction):**

U87, U373, T98G, LN229, & A172 cells were grown and after attaining 70-80% of confluency, total mRNA was extracted using TRIsoIn (GeNei company). 1μg of total RNA was reverse transcribed using iScript cDNA synthesis kit (BIO RAD) following the manufacturer’s protocol. Quantification of cDNA was done by taking OD at 260/280nm using Nanodrop Spectrophotometer. In similar manner the extraction of mRNA was carried out in U87 and T98G cells knock down for hnRNPA1 (V1) and hnRNPA1 (V2) as mention above. Gene was amplified using specific forward and reverse primer (Table 1) with the help of Taq DNA Polymerase in thermocycler (BIO RAD). Quantitative Realtime PCR was carried out in a 20μl reaction volume containing 2 μl cDNA (50ng/μl), 10μl 2x Maxima SYBR Green mix, 1μl each of forward and reverse primers and 6μl of nuclease free water. A control reaction was carried out simultaneously using housekeeping gene (18S or β-Actin) specific primers to compare the expression level. The reaction was carried out in an Applied Biosystems 7300 Fast Real-Time PCR with standard operating procedures. After running of samples, Ct value was obtained. Relative expressions of experimental and control samples were determined by delta Ct method. Each experiment was done and analyzed in triplicate.

\[ \Delta Ct = Ct \text{ of Experimental Gene } - \text{ Ct of Housekeeping Gene} \]
ΔΔCt = ΔCt of Experimental Sample – ΔCt of Control Sample Relative fold change in gene expression = 2-ΔΔCt

Melt curve was analyzed to make sure that the fluorescence signal, from the given acquisition temperature, was contributed only by specific product not by primer dimer.

Qualitative Gene Expression by Polymerase Chain Reaction (PCR) and Agarose Gel Electrophoresis:

Polymerase chain reaction was carried out using Techne Prime Thermal Cycler and Biorad Thermal Cycler with standard operating protocols. The amplified product was run in 1 – 1.5% agarose gel. Gel was viewed and imaged in Gel-Doc instrument.

Statistical Analysis:

Numerical values obtained from individual experiments were expressed here, as mean ± standard error of the mean, unpaired student T-test, p<0.5 were taken in analysis, and represented.

Biinformatic Analysis of ABCC4 and ABCC6

i) RBP Binding Site Prediction:

RBPmap is a webserver, freely accessible through the website http://rbpmap.technion.ac.il/ for prediction of binding sites of RBPs [70]. It is based on a Weighted-Rank approach for mapping the motifs, which regards the tendency of binding site to be clustered and conserved. Major splicing protein motifs were selected from a large database of experimentally defined motifs. The 3’ splice site and 5’ splice site of exon 3 and exon 4 of ABCC4 was used to find the binding sites of splicing factors.

ii) Protein-Protein Interaction Network:
BioGRID (General Repository for Interaction Datasets) is a curated database of non-redundant physical and genetic interactions in dozens of species [71]. It was used to retrieve interactors of ABCC4 and ABCC6 proteins. Visualization of the data as a graph network was done by Cytoscape [72]. BiNGO was used to find GO (Gene Ontology) of proteins, and over-represented GO term in the network relative to all genes or their protein products in the genome [73].

Results:

**ABCC4 and ABCC6 are expressed in human Glioma cell lines**

As reported in various studies, expression of ABCC transporters in human glioma is always conflicting and not consistent. First, we detected the expression pattern of ABCC2, ABCC3, ABCC4, ABCC5 and ABCC6 transporters in human glioma cell lines (U87, U373, LN229, A172, T98G) by quantitative real-time PCR (qRT-PCR). No expression was detected for ABCC2, ABCC3 and ABCC5. The expressions of ABCC4 and ABCC6 were well detected (Fig 3,4,5,6). Relative expressions were plotted using one of the cell lines (cell line with highest Ct value). ABCC4 was least expressing in U373 cell line and highest expression was observed in LN229 followed by T98G and A172. ABCC4 was abundantly expressed in LN229 compared to lowest expressing U373. Similarly, expression for ABCC6 was plotted relative to LN229 (cell line with highest Ct). ABCC6 was expressed least in U373 cell line and highest expressions were observed in T98G followed by LN229 and A172. The expressions were also checked using PCR-Agarose gel electrophoresis (semi-quantitative/qualitative). Similar observations complement the above qRT-PCR findings. Thus, confirming the expression of ABCC4 and ABBC6 in human glioma cell lines. Expression was normalized with the housekeeping gene.

**HnRNPA1 variant 2 knockdown increases the expression of ABCC4**
To check whether hnRNPA1 controls the expression of ABCC4, we analyzed the expression of ABCC4 in siRNA mediated knockdown hnRNPA1 (V1) and hnRNPA1 (V2) human glioma cells (U87 and T98G). No significant difference was observed between U87 Cti and U87 hnRNPA1 (V1i). But, ABCC4 was significantly overexpressed in hnRNPA1 (V2i) sample (3 fold) (Fig 7).

Similarly, upon hnRNPA1 (V2) knockdown in T98G cells, the expression of ABCC4 increased by 18.37-fold compared to the knockdown control (Fig 8). There was no significant effect of hnRNPA1 (V1) knockdown on ABCC4 expression in T98G cells. To further confirm the expressions, we have checked the expression of ABCC4 by PCR – Agarose gel electrophoresis. The expression pattern was similar in agarose gel electrophoresis experiment (Fig 9). Thus, confirming that hnRNPA1 (V2) knockdown increases the expression of ABCC4 in human glioma cells (U87 and T98G) whereas hnRNPA1 (V1) knockdown has no significant difference in ABCC4 expression compared to control. This indicates that ABCC4 expression is inhibited by hnRNPA1 (V2).

**Knockdown of hnRNPA1 V1 and hnRNPA1 V2 decreases the ABCC6 expression**

To investigate the effect of hnRNPA1 (V1) and hnRNPA1 (V2) knockdown on ABCC6 expression, we analyzed the expression of ABCC6 in siRNA mediated hnRNPA1 (V1) and hnRNPA1 (V2) knockdown U87 human glioma cells. We found decreased expression of ABCC6 in hnRNPA1 (V1) knockdown U87 cells (0.48 fold) and also in hnRNPA1 (V2) knockdown U87 cells (0.40 fold) as shown in Fig 8. The expression is further confirmed with PCR - Agarose gel electrophoresis and similar expression profile was observed in both U87 and T98G cells (Fig 10,11). Together with quantitative RT-PCR observations, this data suggests that hnRNPA1 (V1) and hnRNPA1 (V2) regulates the ABCC6 expression.

**Bioinformatic Analysis of ABCC4 and ABCC6**
i) RBP Binding Site Prediction:

For prediction of RBP binding site the splice site sequences from the amplicon of ABCC4 were used as it was highly influenced on hnRNPA1 v2 silencing. The results of binding site prediction were visualized in graphical representation (Fig 12, 13). The 5’ splice site of exon 3 of ABCC4 was predicted to be a binding site of hnRNPA1 with a no. of other splicing proteins. Whereas the 3’ splice site of exon 4 did not have a predicted hnRNPA1 site, but contained binding site for a number of other splicing factors.

ii) Protein-Protein Interaction Network:

Interaction network of ABCC4 and ABCC6 were retrieved from the BioGRID as they were expressed in glioma cell lines and were influenced by hnRNPA1 knockdown. The interaction network formed from 38 published interactions of ABCC4 is reported (Fig 14). The figure is color coded representing a major feature of a group of protein in the network. Fig 15 shows the comparatively smaller interaction network with not much interaction involving ABCC6.

**Discussion:**

ABC subfamily C transporters are well known for multidrug resistance in various cancers [10]. These transporters confer resistance by efflux transport of anticancer drugs and also decrease the sensitivity of drugs [12]. ABCC transporters are overexpressed in various tumors [11]. ABCC1/MRP1 is highly expressed in esophageal cancer, Non-small lung cancers and in leukemias [11], [31] & [32]. ABCC2/MRP2 is overexpressed in hepatocellular carcinoma (HCC) [33]. MRP3 is found highly expressed in acute myeloid leukemia and acute lymphoblastic anemia [34], [35]. MRP4 has been reported to overexpress in primary lung cancers and primary neuroblastoma [36], [37]. MRP5 and MRP6 are highly expressed in Non-small cell lung cancer (NSCLC) [38], [39]. ABCC10/MRP7 is expressed in colorectal cancer and in paclitaxel resistant cells [40], [41].
Similarly, ABCC11/MRP8 is overexpressed in MTA resistance lung cancer [42]. Overexpression of these transporters has been associated with drug resistance in various cancers [11]. Expression of ABCC transporters and drug resistance were not properly investigated in the human glioma. GBM is one of the most aggressive human cancers. The effective treatment and clinical outcome are limited by number of factors including drug resistance [43]. Multidrug resistance in cancer cells is often attributed to ABCC family members [10]. Therefore, it was quite reasonable to investigate the ABCC transporters expression in human glioma.

In the current study we found good expression of ABCC4 in human glioma cell lines. ABCC4/MRP4 is one of the members of the ABCC/MRP subfamily of ABC transporters and its locus is found in human chromosome 13q32 [44]. The full length of the gene is approximately 312kb containing 31 exons and encodes 1325 amino acids [45]. ABCC4/MRP4 is the smallest transporter among its family and its structure is similar to P-glycoprotein (P-gp) [46]. They can pump a wide variety of nucleoside analogs out of the cell, including cGMP, cAMP and anticancer drugs out of cells [47]. They are expressed in almost all human tissues, from kidney to brain and testis except the bone marrow, cervix, thymus, vascular endothelium and soft tissue [48].

Zhao et. al. in their study [49] shown that ABCC4/MRP4 was highly expressed in lung cancer cell lines (A549 & 801D). Suppression of ABCC4/MRP4 expression in this cancer cell lines by RNA interference technique leads to inhibition of cell growth and increase the percentage of cells in G1 phase. They also showed that phosphorylation of retinoblastoma protein (pRB) was weakened when ABCC4/MRP4 expression was inhibited in the cancer cell lines. pRB play in important role in regulating cell proliferation by controlling progression through the restriction point in the G1 phase of the cell cycle [50]. Phosphorylation of pRB results in release of the transcription factor E2F, which induces expression of genes required for progression of the cell cycle [51]. Therefore, pRB may be regulated by ABCC4/MRP4 via transporting cell signal molecules in lung cancer. In pancreatic ductal adenocarcinoma cells (PDAC) higher levels of MRP4 expression tend to have a
shorter overall survival than those with lower levels of MRP4. Inhibition of ABCC4/MRP4 produces an indirect activation of cAMP/EPAC/Rap1 pathway that abrogates cell proliferation and could be an exploitable therapeutic target for PDAC [52].

Zhang et.al. in their study shown that ABCC4/MRP4 gene was highly expressed in multiple types of gastric cancer cells as compare to normal gastric mucosal cells, were its expression is very low or undetectable. Downregulation of ABCC4 in drug-resistant human gastric cancer cells caused translocation of Bax and Bcl-2 and significantly increased caspase-3 and -9 expression levels which play an important role in activation of apoptosis through mitochondrial pathway. Furthermore, in the same study, ABCC4 knockdown in 5-flourouracil (5-FU)-resistant cancer restored 5-FU sensitivity, resulting in the inhibition of cell proliferation and tumor growth in nude mice [53]. Thus, inhibition of ABCC4 gene expression can inhibit the proliferation of MDR gastric cancer and can enhance gastric cancer cell sensitivity to chemotherapeutic drugs through the mitochondrial pathway.

Therefore, in most of the cancers high expression of ABCC4 result in the development of drug resistance and low level of survivability. However, little is known about the function of ABCC4 in human glioma cells. In one of the study inhibitions of ABCC4 produces an indirect activation of cAMP/EPAC/Rap1 pathway that abrogates cell proliferation in PDAC. On the other hand, elevation of intracellular cAMP promotes morphological differentiation and decreases proliferation in cultured neuroblastoma cell lines [54], [55].

Further in the current study we see the role of alternating splicing factor hnRNPA1 in the expression of ABCC4 gene in human glioma cells. We found that knockdown of hnRNPA1 V2 increased the ABCC4 expression. Chromatin immunoprecipitation and luciferase reporter assays demonstrate that MRP4 expression is directly regulated by the MYCN and MYC oncogenes, raising the possibility that MYCN induction of ABCC4 expression may contribute to the malignant phenotype driven by MYCN in neuroblastoma. Oncogenic transcription factor c-Myc upregulates transcription of
hnRNPA1 in human gliomas [56]. Our findings have suggested that hnRNPA1 V2 might play an important role in multidrug resistance associated with ABCC4 (MRP4). Since hnRNPA1 is an important and well-known splice regulator, it might be regulating the splicing of ABCC4. Further, hnRNPA1 is a well-studied member of ubiquitously expressed hnRNP family. It is involved in pre-mRNA processing, mRNA metabolism and transport. HnRNPA1 V2 might be regulating ABCC4 mRNA processing and metabolism.

In another study, ABCC6 expression is decreased upon hnRNPA1 (V1) and hnRNPA1 (V2) knockdown. This suggests that both isoforms of hnRNPA1 might be promoting ABCC6 expression directly or indirectly. HnRNPA1 also regulates genes involved in tumorigenesis and malignant transformation. Since, ABCC6 expression is associated with multidrug resistance by cancer cells, hnRNPA1 might be regulating ABCC6 expression and promotes tumor survival.

The drug-resistant cell lines have over-expression of hnRNPA1 (V2) [unpublished data]. Whereas the cell lines used in our studies were non-resistant and hnRNPA1 (V2) should have a limited expression in them. Therefore, it is possible that further knockdown of hnRNPA1 v2 led to over-expression of ABCC4 not by direct influence but through some other regulatory control on the ABCC4 pre-mRNA. To test this possibility, we require more experimental data related to the ABCC4 pre-mRNA stability in hnRNPA1-v1 and v2 knockdown samples in resistant and non-resistant glioma cell lines.

Through the interaction network study, we found several interactors of ABCC4, which have a functional role in modulating ABCC4. The interaction between ABCC4 and MPP1 (Membrane Palmitoylated Protein 1) was reported to increase membrane localization and retention of ABCC4 and subsequently enhanced drug resistance in AML (Acute myeloid leukemia) [57]. Protein binding partners like SLC9A3R1 and SNX27 have a significant role in internalization and cell surface expression of ABCC4 [58],[59]. LGAL3 (Galectin 3) a carbohydrate-binding protein evidenced to be physically interacting with ABCC4, is known to be involved in the progression of cancer cells.
and apoptosis resistance [60], [61]. Interestingly, ABCC4 has been found to interact with HAX-1 (Hematopoietic-substrate-1 associated protein X-1) in high throughput study [62]. HAX-1 is an anti-apoptotic protein reported in many studies to play an essential role in several malignant tumors [63]. Another interactor of ABCC4 is the Lysophosphatidic Acid Receptor 1 (LPAR1) [64]. LPAR1 acts as the primary mediator responsible for LPA-stimulated metastasis in various cancers [65], [66]. Among the interactor of ABCC6, NR4A1 plays a significant role in acting as a pro-oncogenic protein and promotes cancer invasion and metastasis in tumors [67],[68]. While some of these proteins are already being tested and used as targets for cancer therapy, future research on the biological effect of these interactions could potentially extend the explanation of the role of ABCC proteins in cancers.

In conclusion, ABCC4 and ABCC6 are well expressed in human glioma cells and might be contributing to drug resistance. HnRNPA1 (V1) and hnRNPA1 (V2) regulates the ABCC4 and ABCC6 expression. Thus, might play important role in drug resistance.

Our study found good expression of ABCC4 and ABCC6 in human glioma cells. As per our understanding of drug resistance by ABCC transporters, we suggest that ABCC4 and ABCC6 might be associated with drug resistance in glioma. Further studies on protein expression and drugs efflux are required to confirm these findings.

**Declaration:** No conflict of Interest

**Acknowledgement:** Thanks for receiving support from various Funding agency such as; SERB-New Delhi (India), DST-PURSE support from University of Delhi. Research & Maintenance Grant from University of Delhi.
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Figures and Tables:

| Primer | Order   | Sequence (5’ to 3’)                      | Amplicon Size (bps) |
|--------|---------|------------------------------------------|---------------------|
| ABCC 2 | Forward | GGATGCACAAAAAGGCCTTCA                    | 164                 |
|        | Reverse | GTCGAATGGCAGATGTGTCC                     |                     |
| ABCC 3 | Forward | ACCAACTCAGTCAAACGTGC                    | 194                 |
|        | Reverse | AGACCATGAAAGCGACTCCA                     |                     |
| ABCC 4 | Forward | GAGCTGAGAATGACGCACAG                    | 196                 |
|        | Reverse | TACGCTGTGTTCAAAGCCAC                     |                     |
| ABCC 5 | Forward | GTGCCTTAGATGCCCATGTG                    | 163                 |
|        | Reverse | TGGGTGCCTCTTTTCGTAAT                     |                     |
| ABCC 6 | Forward | CTGTCTCCAAGCCATTGGGC                    | 517                 |
|        | Reverse | AGCCACCAGTCGCGGAAAC                     |                     |
| 18SrRNA | Forward | GCAATTATTCCCATGAACG                    |                     |
|        | Reverse | GGACTTTAATCAACGCAAGC                    |                     |
**Figure 1:** Schematic diagram of primer position on ABCC4 pre-mRNA

**Figure 2:** Schematic diagram of primer position on ABCC6 pre-mRNA
Figure 3: Relative expression of ABCC4 in glioma cell lines (U87, U373, LN229, A172, and T98G). N-fold Expression is represented relative to U373.
Figure 4: Qualitative expression of ABCC4 in human glioma cell lines (U87, U373, LN229, A172, and T98G) using PCR-Agarose gel electrophoresis system. Amplicon size of ABCC4 is 196bp.
Figure 5: Relative expression of ABCC6 in glioma cell lines (U87, U373, LN229, A172, and T98G). N-fold Expression is represented relative to LN229.
Figure 6: Qualitative expression of ABCC6 in human glioma cell lines (U87, U373, LN229, A172, and T98G) using PCR-Agarose gel electrophoresis system. Amplicon size of ABCC6 is 517bp.
Figure 7: Expression of ABCC4 in hnRNPA1 v1 knockdown and hnRNPA1 v2 knockdown U87 human glioma cells. HnRNPA1 v1 knockdown decreases ABCC4 expression by 0.48-fold (P<0.0008). HnRNPA1 v2 knockdown increases ABCC4 expression by 3.19-fold (P<0.003). Statistical data were analyzed by t-tests.
Figure 8: Expression of ABCC4 in hnRNPA1 v1 knockdown and hnRNPA1 v2 knockdown T98G human glioma cells. HnRNPA1 v1 knockdown increases ABCC4 expression by 1.90-fold. HnRNPA1 v2 knockdown increases ABCC4 expression by 18.37-fold.

Figure 9: Expression of ABCC4 in hnRNPA1 v1i and hnRNPA1 v2i knockdown U87 and T98G cells with PCR - Agarose Gel Electrophoresis. Amplicon size of ABCC4 is 196bp.
Figure 10: Expression of ABCC6 in hnRNPA1 v1 knockdown and hnRNPA1 v2 knockdown U87 human glioma cells. HnRNPA1 v1 knockdown decreases ABCC6 expression by 0.45-fold (P<0.003). HnRNPA1 v2 knockdown decreases ABCC4 expression by 0.62-fold. Statistical data were analyzed by t-tests.

Figure 11: Expression of ABCC6 in hnRNPA1 v1 and hnRNPA1 v2 knockdown U87 and T98G cells with PCR-Agarose Gel Electrophoresis. Amplicon size of ABCC4 is 517bp.
Fig. 12. Prediction of RBP binding site on 5’ splice site of exon 4 of ABCC4. Shows the presence of cis-regulatory sequence for hnRNPA1 binding on 5’ splice site of ABCC4.

Fig. 13. Prediction of RBP binding site on 3’ splice site of exon 3 of ABCC4.
Figure 14: Interaction network of ABCC4 consisting of known physical protein-protein interactions retrieved from BioGRID.

Figure 15: Interaction network of ABCC6 consisting of known physical protein-protein interactions retrieved from BioGRID.