Bioinformatic Analysis Identifying S100a9 Associated With Cell Proliferation and Metastasis in Neuroblastoma

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Abstract

Background: More than half of neuroblastoma (NB) patients presented with distant metastases and the mortality of patients suffering from metastatic relapse was about 90%. It is urgent to find a biomarker that can facilitate the prediction of metastasis in NB patients.

Methods and Results: In the present study, we systematically analyzed Gene Expression Omnibus (GEO) datasets and focused on identifying the critical molecular networks and novel key hub genes implicated in NB. We found totally, 176 up-regulated and 19 down-regulated differentially expressed genes (DEGs) were identified. Based on these DEGs, a PPI network composed of 150 nodes and 452 interactions was established. PPI network identification combined with qRT-PCR, ELISA and IHC, S100A9 as was screened as an outstanding gene. Furthermore, in vitro tumorigenesis assays demonstrated that S100A9 overexpression enhanced the proliferation, migration, and invasion of NB cells.

Conclusions: Taken together, our findings suggested that S100A9 could participate in NB tumorigenesis and metastasis and that S100A9 has the potential to be used as a biomarker in the prediction of NB metastasis.

Introduction

Neuroblastoma (NB), the most aggressive form of solid tumor of infants, account for 15% of cancer deaths in children.[1,2] For NB patients in high-risk, the main preferred treatment choices remain chemotherapy and radiotherapy, which lead to tremendous toxicity and drug resistance inevitably, , the cases ratio to recurrence and progression is about 50%[3]. Thus, it is urgent for us to identify new effective biomarker for early diagnosis, metastasis prediction and ideal therapeutic target for NB patients.

S100A9, which is also called calgranulin B or migration inhibitory factor-related protein 14 (MRP-14), belongs to the low-molecular-weight calcium-binding S100 protein family. This family comprises more than 20 members. [4] Many evidences show S100A9 is correlated with tumorigenesis, immunofluorescence analysis has shown that S100A9 protein is elevated in metastatic melanoma and prostate cancer [5,6]. In these tumors, increased expression of S100A9 was correlated with poor differentiation. In both malignant and surrounding non-malignant tissues, a huge number of inflammatory cells expressing S100A9 were associated with significantly shorter cancer survival.[7]. while downregulated the expression of S100 family members is correlated with tumor proliferation, inflammation invasion, and angiogenesis.[8] Although S100A9 have been studied in many types of cancers[9] [10-13],but their biological functions in NB malignancies remain poorly understood.

In the present study, based on the Geo database[14], bioinformatic analysis was further performed. After screening of DEGs and functional analysis, PPIs of DEGs were analyzed. And we found that the expression of S100A9 is pretty high in NB patients. In addition, we investigated the biological functions of S100A9 in NB cell line, as well as in primary human NB samples. These results may identify new effective biomarker for early diagnosis, metastasis prediction and ideal therapeutic target for NB patients, and provide valuable biological information for further investigation of NB.

Materials And Methods

Bioinformatic analysis

Data processing and analysis of gene expression profile

The public microarray dataset GSE90121, which was obtained based on the Affymetrix GPL570 platform Affymetrix Human Genome U133 Plus 2.0 Array), was downloaded from the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>), [14]. This dataset was deposited by Kaplan D et al., containing information from human NB SK-N-AS metastatic subpopulations isolated after in vivo selection, aimed to identify genomic signatures that regulate metastasis and candidate therapeutics for NB patients. A total of 16 samples were included in the current dataset, including 12 metastatic samples and 4 primary samples. Robust Multi-array Average (RMA) affy package of Bioconductor was used to adjust the raw data. The processed gene expression data was then filtered to include those probe sets with annotations which reference the new version annotation files. To identify DEGs, we used the Linear Models (Microarray Data package in Bioconductor) to compare the expression levels of genes between the metastatic group and the localized tumor group[15].

An adjusted p-value of <0.05 and a |log2FC (fold change) | of ≥ 2 was used as the threshold.

Functional enrichment analysis of DEGs

Database for annotation, visualization and integrated discovery (DAVID) integrates a set of functional enrichment tools to distinguish functional genes underlying diseases processing (<http://david.abcc.ncifcrf.gov/>)[16]. GO (Gene Ontology) function and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analyses of DEGs were performed based on DAVID. P value < 0.05 and count ≥ 2 was regarded as statistically significant differences.

Protein–protein interaction network construction by STRING

We used the Search Tool for the Retrieval of Interacting Genes (STRING), an online tool and biological database for prediction of interactions between proteins, to construct the PPI network [17]. According to our analysis based on STRING, score=median confidence ≥ 0.15 was the standard of PPIs of DEGs selections. The Cytoscape software was used to visualize the PPI network.[18]. The proteins that have many interaction partners constitute the extremely important nodes in the PPI network. These proteins were defined as the hub proteins in this study. To identify such hub proteins in the PPI network, we utilized six bioinformatic tools, namely Closeness, Degree, EPC, MNC, Radiality, and Stress centrality. Sub-network analysis was then conducted to help us discover the outstanding genes.

NB patients, tissue samples and cell lines

Primary tumor tissues were obtained from 9 NB patients with bone marrow metastasis and 10 NB patients without bone marrow metastasis who had undergone tumor resection surgery at the Affiliated Hospital of Qingdao University. None of the included patients was treated with chemotherapy, hormonal therapy or radiotherapy before the tumor resection surgery. Written informed consent was obtained from all the participants. The current research was conducted with the permission of the Medical Ethical Committee of Affiliated Hospital of Qingdao University (Qingdao, China).

Human NB cell line SH-SY5Y was kindly provided by Professor Xiao from the Guizhou Medical University. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) contained 10% fetal bovine serum (FBS, Hyclone, USA) under the conditions of 37°C, 5% CO₂.

NB serum samples and Quantitative real-time PCR

The expression of *TAC1*, *PTGS2* and *FGF1* was examined by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). The collected tissues were immediately frozen at -80°C after surgery. Total RNA was extracted from cancer tissues by TRIzol reagent (Invitrogen Life Technologies). Then the extracted RNA was transcribed into cDNA using random primers and analyzed with an ABI 7000 Real-Time PCR System (Applied Biosystems). PCR primers were as follows: TAC1 primers: (forward) 5'-TGA TCT GAA TTA CTG GTC CGA CT-3' and (reverse) 5'-TCC GGC AGT TCC TCC TTG A-3'; PTGS2 primers: (forward) 5'-TAA GTG CGA TTG TAC CCG GAC-3' and (reverse) 5'-TTT GTA GCC ATA GTC AGC ATT GT-3'; FGF1 primers: (forward) 5'-CTC CCG AAG GAT TAA ACG ACG-3' and (reverse) 5'-GTC AGT GCT GCC TGA ATG CT-3'; GAPDH primers (forward) 5'-CAG CGA CAC CCA CTC CTC-3' and (reverse) 5'-TGA GGT CCA CCA CCC TGT-3'. Reactions were performed in triplicate using SYBR Green master mix (TaKaRa, Japan) and normalized to GAPDH mRNA level using the $\Delta\Delta Ct$ method.

ELISA

To quantify levels of S100A9 in plasma, ELISA was performed as previously described [19] By using human S100A9 (JYM0539Hu, JYM, China) ELISA kits, S100A9 in plasma of the NB patients were detected according to the manufacturer's recommended procedure.

IHC staining

In brief, the formalin fixed, paraffin-embedded tissues sections were deparaffinized, rehydrated and boiled in 0.01 M citrate buffer for 10 min, then incubated with 0.3% H₂O₂ in methanol to block endogenous peroxidase activity. Then the sections were incubated with the anti-S100A9 antibody (Cell Signaling Technology, USA), followed by incubation with secondary antibody tagged with the peroxidase enzyme for 30min at room temperature, finally visualized with 0.05% DAB (3,3'-diaminobenzidine) until the desired brown reaction product was obtained. All slides were observed under a OLYMPUS BX41 Microscope, and representative photographs were taken.

Construction of plasmids and establishment of stably transfected cells

SBI-piggyBac vector, GST-S100A9, were kindly provided by Professor T.C. He from the University of Chicago. To construct an S100A9 overexpression plasmid, the complete coding sequence of human S100A9 gene was subcloned into the SBI-piggyBac vector. For S100A9 silencing, siRNAs targeting human S100A9 with the sequences of 5'-GCAAGACGAUGACUUGCAA-3' and 5'-UUGCAAGUCAUCGUCUUGC-3' were synthesized and assembled into the SBI-piggyBac vector, resulting in SBI-siS100A9. After transfecting SH-SY-5Y NB cells with the constructed plasmids, the stably transfected cells were selected by incubation with puromycin for one week. The stably transfected cell lines, namely Control (SH-SY5Y transfected with SBI empty vector), S100A9(SH-SY5Y transfected with SBI-S100A9) and siS100A9(SH-SY5Y transfected with SBI-siS100A9) briefly.

Cell viability assay

The viability of SH-SY5Y cells was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, stably transfected SH-SY5Y cells were seeded in 96-well plates (1000 cells/well). The cells were incubated in DMEM supplemented with 1% FBS for 24, 48, 72, 96 and 120 h, then incubated with MTT reagent (Progema, Madison, WI, USA, 20 μ L / well) for another 4 h at 37°C to allow the formation of formazan. After that, 100 μ L of dimethyl sulfoxide was added into the cell culture medium for another 10-min incubation at room temperature. At last, in every day of the next five days, a microplate reader (Bio-rad, iMark) was used to measure the absorbance at 492 nm of each well. The experiment included three independent replicates for each sample.

Colony formation assay

Exponentially growing stably transfected SH-SY5Y cells were seeded at a low density (100 cells/well) in cell culture medium containing 1% FBS in 6-well plates. The cells were allowed to grow for about 10 days to form colonies. The culture medium was refreshed every 3-4 days. Crystal violet was used to stained the colonies. Colony numbers from 3 wells were used to calculated the average colony number.

Scratch wound healing assay

The scratch wound healing assay was performed as described previously [20,21]. Briefly, stably transfected SH-SY5Y cells were seeded in 6-well plates and grown to ~90% confluency. Then, sterile micro-pipette tips were used to scratch the monolayer formed by SH-SY5Y cells to create a wound. After that, the medium (DMEM containing 1% FBS) was refreshed every day to remove the floating cells. Bright field microscopy was used to monitor the wound healing status at 24 h, 48 h, and 72 h after the wound was created. Each assay was repeated three times. imageJ software was used to calculate the wound healing ratio.

Transwell invasion assay

A chamber coated with non-type I-collagen (Millipore, USA) was used for the Transwell assay. The upper chamber coated with ECM gel (Sigma, USA) was filled with 400 μ L of serum-free DMEM and seeded with exponentially growing stably transfected SH-SY5Y cells (1×10^4 cells). The lower chamber was filled with 500 μ L of DMEM supplemented with 20% FBS, which served as a chemoattractant. After 24 h of incubation, the cells migrated across the Transwell membrane were dried, fixed with methanol, and then stained with hematoxylin-eosin (H&E). Cotton swabs were used to remove the cells on the upper surface of the Transwell membrane. At last, an inverted microscope ($\times 100$ magnification) was used to count the number of cells migrated across the Transwell membrane. Five randomly-selected fields were examined to obtain the mean value of the number of cells migrated across the Transwell membrane. The experiment was repeated three times.

Statistical analysis

All data are presented as means $\pm$ standard deviations. T test was performed in the GraphPad Prism software to determine the statistical significance of differences between groups. A P value of less than 0.05 was considered statistically significant.

Results

Enrichment analyses of DEGs

In general, 176 up-regulated DEGs and 19 down-regulated DEGs were identified based on the cut-off criteria (P -value < 0.05 and count ≥ 2), which were used to generate a heatmap for the NB patients with and without metastasis (Fig. 1A). Functional enrichment analysis revealed that the top 18 mostly enriched GO terms and top 2 mostly enriched KEGG pathways were associated with regulation of nucleosome assembly, innate immune response in mucosa, extracellular matrix organization, angiogenesis and innate immune response, etc. (Table 1).

Table 1
The tops enriched GO terms of the differentially expressed genes

Category	ID	Term	p-value	Gene count	Genes
BP	GO:0001558	regulation of cell growth	3.13E-04	6	NANOS1, NRG3, IGFBP2, AGTR1, IGFBP7, WISP1
	GO:0006954	inflammatory response	5.07E-04	11	CCL7, TRIL, C3AR1, FPR3, IL1RAP, CD01, PTGS2, TAC1, HDAC9, S100A9, CHST2
	GO:0007204	positive regulation of cytosolic calcium ion concentration	0.00316801	6	AGTR1, C3AR1, ADM, FPR3, LPAR3, TAC1
	GO:0030335	positive regulation of cell migration	0.011814659	6	CCL7, GPNMB, CDH13, SEMA3E, LAMC2, FGF1
	GO:0051384	response to glucocorticoid	0.012309797	4	IGFBP2, ADM, CD01, PTGS2
	GO:0007200	phospholipase C-activating G-protein coupled receptor signaling pathway	0.012827699	4	HTR1D, AGTR1, C3AR1, FPR3
	GO:0051482	positive regulation of cytosolic calcium ion concentration involved in phospholipase C-activating G-	0.018038462	3	GPR65, AGTR1, LPAR3
	GO:0008285	negative regulation of cell proliferation	0.027623627	8	MYOG, KAT2B, GPNMB, CDH13, IGFBP7, ADM, PTGS2, MYO16
	GO:0030819	positive regulation of cAMP biosynthetic process	0.035152385	3	GPR65, RLN2, ADM
	GO:0007267	cell-cell signaling	0.040239268	6	CCL7, ADM, TAC1, PCSK5, S100A9, WISP1
CC	GO:0043005	neuron projection	0.007369421	7	SLC32A1, CASP5, PTPRO, STMN2, CDH13, PTGS2, POU4F1
	GO:0005615	extracellular space	0.007817435	19	GDF10, IGFBP2, SECTM1, ADM, LAMC2, SEMA3E, FGF1, PCSK5, WISP1, CCL7, NRG3, PLBD1, CDH13, IGFBP7, TAC1, S100A9, ANGPTL1, MICA, CPA4
	GO:0005576	extracellular region	0.011007958	21	OLFML2B, IGFBP2, PSG1, ADM, LAMC2, SEMA3E, IL1RAP, FGF1, THBS2, FBLN2, APOLD1, CCL7, NRG3, RLN2, FDCSP, IGFBP7, CCDC3, VIP, TAC1, S100A9, CHGB
	GO:0005887	integral component of plasma membrane	0.012602605	19	GABRB1, VIPR1, SLC10A1, GPR65, PTPRO, HTR1D, LPAR3, FPR3, IL1RAP, HCRTR1, TSPAN13, GPNMB, NRG3, GPR183, C3AR1, AGTR1, LGR5, MICA, SLC38A4
	GO:0005901	caveola	0.079588673	3	SDPR, CDH13, PTGS2
	GO:0008201	heparin binding	0.007960505	6	CCL7, GPNMB, LAMC2, THBS2, FGF1, WISP1
MF	GO:0003705	transcription factor activity, RNA polymerase II distal enhancer sequence-specific binding	0.014352101	4	MYOG, FOXC1, POU4F1, VGLL3
	GO:0005179	hormone activity	0.03603451	4	RLN2, ADM, VIP, CHGB
Category	ID	Term	Gene symbol	p-value	

Category	ID	Term	p-value	Gene count	Genes
KEGG_PATHWAY	hsa04080	Neuroactive ligand-receptor interaction	GABRB1, VIPR1, HTR1D, AGTR1, C3AR1, FPR3, LPAR3, HCRT1	9.46E-04	
	hsa04512	ECM-receptor interaction	LAMC2, THBS2, ITGA9	0.090573261	

In addition, volcano plot was able to quick identify the expression changes within the gene sets by combination of statistical tests (adjusted p-value) and magnitude of change. (Fig. 1B)

PPI network analysis

To identify the potential biomarkers that can predict metastasis in NB patients, the DEGs were delineated to construct a PPI network. A DEG with a combined score (median confidence) > 0.15 was regarded as a significantly differentially expressed gene. As shown in Fig. 2A, the PPI network constructed by STRING is composed of 150 nodes and 452 interactions. The Cytoscope software was used to visualize the PPI network. The top 10 hub proteins in the PPI network determined by Closeness, Degree, EPC, MNC, Radiality, and Stress centrality are listed in Table 2. The results showed that TAC1, PTGS2 and FGF1 were the most outstanding genes and maybe play an important role in the NB metastasis. Sub-network analysis suggested that S100A9 proteins were the outstanding hub proteins (Fig. 2B).

Table 2

Top 10 genes that evaluated by betweenness, BottleNeck, Closeness, Clustering Coefficient, Degree ,DMNC ,EcCentricity ,EPC,MCC ,MNC,Radiality and Stress in the protein-protein interaction network

Gene name	Closeness Centrality	Gene name	Degree Centrality	Gene name	EPC Centrality	Gene name	MNC Centrality	Gene name	Radiality Centrality	Gene name
HDAC9	70.66667	SLC22A15	25	TRIM9	22.245	TAC1	25	TAC1	4.87121	
PTGS2	70.08333	ZNF439	23	GPR183	22.121	STMN2	23	FGF1	4.86364	
TAC1	69.75	UBASH3A	20	AK5	22.046	SLC32A1	23	PTGS2	4.84091	
KAT2B	66.41667	CHST2	19	CASQ1	19.554	RGS7	18	TAGLN	4.79545	
FOXC1	66.25	SEMA3E	18	WISP1	19.156	PTGS2	17	CDH13	4.7803	
FGF1	65.83333	HCRT1	17	RGS7	19.142	FGF1	17	AGTR1	4.72727	
STMN2	65.75	GPR183	17	NRG3	18.906	CHGB	16	STMN2	4.7197	
MYOG	64.41667	FPR3	17	ANGPTL1	18.77	IGFBP7	14	MYOG	4.69697	
CDH13	63.45	CCL7	16	SEMA3E	18.362	THBS2	14	KAT2B	4.68939	
TAGLN	62.83333	NRG3	15	GPR65	17.728	NRG3	13	SLC32A1	4.68182	

Validation of the outstand gene in NB patients

To verify the expression of *TAC1*, *PTGS2*, *FGF1* in microarray data, qPCR was performed to detect the expression of those three genes, finally, the qRT-PCR results showed that the expression of three gene there were no significant differences between the 9 NB patients with bone marrow metastasis and 10 NB patients without bone marrow metastasis (Fig. 3A). Next, S100A9 identified in the microarray data, ELISA and IHC were performed to detect the protein levels of S100A9 in NB patients (9 NB patients with bone marrow metastasis and 10 NB patients without bone marrow metastasis). The serum levels of S100A9 were higher from NB patients with bone marrow metastasis than that from NB patients without bone marrow metastasis. (Fig. 3B) Further, we also examined the expression of S100A9 in sections from NB patients with bone marrow metastasis tissues and NB patients without bone marrow metastasis using IHC staining (Fig. 3C). The results showed that the expression levels of S100A9 were significantly higher in NB patients with bone marrow metastasis than from NB patients without bone marrow metastasis.

S100A9 overexpression promoted the proliferation, migration and invasion of SH-SY5Y cells

To investigate the effects of *S100A9* on the proliferation of SH-SY5Y cells, the coding sequence of human *S100A9* gene expressed in GST-S100A9 vector was subcloned into the SBI-piggyBac plasmid to overexpress *S100A9* in SH-SY5Y cells. According to the MTT assay results, the SH-SY5Y cells overexpressing *S100A9* (defined as the S100A9 group) exhibited higher proliferation ability than the SH-SY5Y cells transfected with empty vector (defined as the control group) at days 3, 4, and 5 (P < 0.05). The S100A9-knockdown SH-SY5Y cells (defined as the siS100A9 group) exhibited significantly slower proliferation when compared with the control group at days 3, 4, and 5 (all p values < 0.001) (Fig. 4A). Colony formation assay showed that the S100A9 group formed more

colonies compared with the control group. Quantitatively, the number of colonies formed in the S100A9 group was approximately double than that of the control group (Fig. 4B). These results indicated that *S100A9* overexpression accelerated the proliferation of SH-SY5Y cells. In addition, the results showed that the migration, and invasion of SH-SY5Y cells were significantly active by S100A9, as revealed by Transwell assays (Fig. 5A), wound healing (Fig. 5B).

Discussion

In the current study, the GSE90121 dataset which was deposited by Kaplan D. were downloaded and analyzed by bioinformatics method to identify potential crucial genes associated with NB metastasis. A total of 195 genes including 19 down-regulated and 176 up-regulated genes were obtained. Besides, the significantly enriched GO terms were mainly focused in regulation of cell growth, inflammatory response, positive regulation of cell migration. Hub genes of the regulatory network were then selected and conducted with PPI network module. Dysregulated *TAC1*, *PTGS2* and *FGF1* were the top 3 outstanding genes based on both six methods (Closeness, Degree, EPC, MNC, Radiality, and Stress centrality) evaluation. The expression levels of *TAC1*, *PTGS2* and *FGF1* in resected specimen of NB patients with or without metastasis were then validated by qRT-PCR, although the expression of *TAC1*, *PTGS2* and *FGF1* were related with many kinds of tumorigenesis, such as non-small cell lung cancer[22], pancreatic ductal cancer[23], colorectal cancer[24–26], squamous cell carcinoma[27], gastric cancer[28] and clear cell renal cell carcinoma[29], but these genes expression did not exhibit significant change as expected as the microarray results, indicating that these three genes may not be the pivotal gene that participate in the metastasis of NB.

After go through the differentially expressed genes list and reviewing the relevant literatures, we found that S100A9 exhibits a broad range of biological functions involving in various cancer progression[30–32], specifically, S100A9 proteins take part in a broad range of intracellular and extracellular functions by regulating calcium balance, cell apoptosis[33], migration[34], proliferation [35], differentiation[36, 37, 9], and inflammation[38] But fewer research investigated the expression and biological function of S100A9 in NB cells. Here, the promoted expression levels of S100A9 were verified to be consistent with the microarray results. To further validate the results, we observed that elevated S100A9 promoted the proliferation and migration of NB cancer cells. These findings suggested that S100A9 may be an important carcinogenic factor in the occurrence and progression of NB and may serve as an attractive therapeutic target for NB patients.

Conclusions

Collectively, the novel potential biomarker S100A9 was identified by bioinformatics methods in the present study. And our results suggested that S100A9 promote the proliferation and migration of NB cell and may play a pivotal role in NB metastasis and expected to get a further insight into the molecular mechanisms of NB patients and would help us discriminate the metastatic possibility of NB patients.

Declarations

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval This study was approved by the Medical Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology.

Consent to participate Informed consent were obtained from the participants or their parents. Consent for publication All authors had gone over the manuscript and consent to submit

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Author Contributions

YYZ conceived the study. XC, YKX, JF, QWT and QW perform the study. Statistical analysis was undertaken by XC. All authors read and approved the final version of the manuscript. QW and YYZ wrote the manuscript.

Data Availability

The following information was supplied regarding data availability: The data is available at NCBI GEO: GSE90121

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Figures

Figure 2

Protein-protein interaction network constructed with the up- and down-regulated differentially expressed genes.

Figure 3

PPI network was visualized by Cytoscope software.

Figure 4

(A). qRT-PCR analysis for the expression of hub genes. (B). ELISA analysis for serum levels of S100A9 in metastatic NB (n=9) and non-metastatic NB(n=10). (C). Representative IHC staining for S100A9 in tissue sections from metastatic NB and non-metastatic NB. **p < 0.01.

A

B

Figure 5

(A). MTT analysis for Blank/Control/S100A9/siS100A9 SH-SY-5Y cells for sequential 5 days. * $P < 0.05$, ** $p < 0.01$ (B). Colony formation assay for Blank/Control/S100A9/siS100A9 SH-SY-5Y cells. The representative images of transmembrane cells are shown in the right panel, the mean numbers of transmembrane cells $\pm$ SD per microscopic field of three independent experiments are quantified in the right panel. ** $p < 0.01$, *** $p < 0.001$