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Decreased invasion ability of hypotaurine synthesis deficient glioma cells was partially due to hypomethylation of Wnt5a promoter

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Abstract: Glioma is one of the lethal central nervous system tumors. The infiltrative and invasive growth nature makes it difficult to identify the boundary between glioma and the normal tissues, resulting in inevitable recurrence after surgery operation. Gliomas do not metastasize, so to prevent the residual tumor from proliferating or invading is a key challenge. Previous report indicated that hypotaurine could facilitate glioma invasion and suppress demethylases' activities. Using a hypotaurine synthesis deficient U251 cell line, we proved that the cells invasion ability was impaired. Gene expression profile analysis exhibited that knocking down one of the key enzymes of hypotaurine synthesis, 2-aminoethanethiol dioxygenase (ADO), significantly affected the extracellular matrix-receptor process. Of that process, Wnt5a expression was severely upregulated by decreased intracellular ADO expression. Cells cultured at the presence of hypotaurine showed a decrease in intracellular Wnt5a protein and mRNA levels. This phenotype was due to hypermethylation of Wnt5a promoter, which was most likely the result of hypotaurine's inhibiting demethylases activities. Collectively, this study demonstrated that hypotaurine synthesis deficient U251 cells were prone to epigenetic modification and Wnt5a seemed to be a tumor suppressor under that circumstance. This tumor suppression effect is warranted to be reevaluated in real tumor samples and the relevant evidence might contribute to develop new glioma interference strategies.

Introduction

Glioma, although with its lower incidence, is the most lethal nervous system malignance worldwide (Wesseling and Capper, 2018). Roughly, WHO classified gliomas into 4 grades (I-IV) and the higher the tumor grades, the stronger the tumor's invasiveness (Cohen *et al.*, 2013). Due to its infiltrative growth nature, the boundary of glioma and the normal tissues is not clear. To data, complete surgical removal of the pathological tissue is still a challenge. Owing to the existence of the residual tumor tissues, post-operation recurrence is nearly inevitable even with extensive adjuvant chemotherapy. Once the relapse occurs, the prognosis is usually worse and over 95% of the recurrent patients will die within 3 years (Krex *et al.*, 2007).

Seldom could we find the metastasis of gliomas. The nature of local infiltrative and invasive growth is the key feature of glioma. Many mechanisms had been studied to elucidate the invasion ability of gliomas. Most of the studies focused on the abnormal activation or inhibition of proteins belonging to certain signal pathways, e.g. mammalian target of rapamycin complex (Zhang *et al.*, 2018), the Wingless/Int1 (Wnt) proteins (Mehta and Lo Cascio, 2018) and transforming growth factor-β (Rodon *et al.*, 2014). Those pathways had close relationship with metastasis and invasion across different types of malignant tumors. Most of the dysregulated activation of the relevant pathways had the background of genetic abnormalities.

Except the protein molecules, much attention has been paid to the functions of small molecular metabolites

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in regulation glioma invasion. In most of the lower-grade and some of the grade-IV gliomas (glioblastoma, GBM), isocitrate dehydrogenase 1 (IDH1) and IDH2 are commonly found mutated (Cohen *et al.*, 2013). Normally, IDH catalyzes isocitrate to generate α-ketoglutarate (α-KG). The loss-of-function mutation of IDHs in gliomas results in the massive yield of α-hydroxyglutarate (α-HG) instead of α-KG from the breakdown of isocitrate (Dang and Su, 2017). α-KG is the natural substrate of many α-KG-dependent dioxygenases. Some of the dioxygenases involve in epigenetic modification of the genome such as 5-methylcytosine hydroxylases and histone lysine demethylases (Medeiros *et al.*, 2017). Due to the structure similarity between α-HG and α-KG, the former can compete off the binding of the latter to the relevant dioxygenases, resulting in suppression of the relevant enzymatic activities. This suppression usually brings about a hypermethylation signature of the genome and contributes to glioma's invasion to varied extent (Flanagan *et al.*, 2012; Jalbert *et al.*, 2017).

Recently, hypotaurine, a sulfur-containing amino acid, was reported to be elevated in glioma tissues (Gao *et al.*, 2016). Its intracellular content correlated to tumor grades apparently. Of note, hypotaurine could competitively inhibit the binding of α-KG to many dioxygenases such as 5-methlycytosine (5mC) hydroxylase, histone demethylase (H3K9) and histone demethylase (H3K4) with the similar mechanism of α-HG (Gao *et al.*, 2017; Gao *et al.*, 2016). Hypotaurine synthesis deficient glioma cells showed compromised invasion ability, which could be rescued by extra hypotaurine supplying (Gao *et al.*, 2016). In order to elucidate the potential mechanism of hypotaurine's effects on invasion, a hypotaurine synthesis deficient glioma cell line was employed in this study. Gene expression profile analysis indicated that the expression of Wnt5a was inhibited by hypotaurine. Furthermore, with the elevated intracellular hypotaurine, promoter methylation status of Wnt5a was enhanced. Thus, we concluded that hypotaurine could inhibited the demethylases activities, resulted in Wnt5a promoter hypermethylation and enhanced the invasion ability of glioma cells.

Material and methods

Reagents

Cysteamine, puromycin, hypotaurine and 5-aza-2'-deoxycytidine were purchased from Sigma-Aldrich (Shanghai, China). Dimethyl sulfoxide (DMSO) was provided by Solarbio (Beijing, China). 5-aza-2'-deoxycytidine was dissolved in DMSO and stored in liquid nitrogen. Cells cultured with 5-aza-2'-deoxycytidine (5 μM) would last for 3 days. The fresh 5-aza-2'-deoxycytidine media were replaced every day.

Hypotaurine synthesis deficient cells

2-aminoethanethiol dioxygenase (ADO) gene knockdown U251 cells (ΔADO) constructed through RNA interference were commercially acquired from 3DMed Shanghai, China. The culture media were DMEM high glucose with 10% FBS and 0.5 μg/ml puromycin. The cells were cultured at 37°C with 5% CO_2.

Quantitative real-time PCR

The cells were cultured overnight to approach 70-80% confluence. Total RNA was isolated using RNAiso plus from TaKaRa Dalian, China. First strand cDNA was synthesized by PrimeScript RT reagent kit with gDNA Eraser (TaKaRa). Quantitative real-time PCR was conducted on Agilent Mx3000P system (Santa Clara, CA) using SYBR Premix Ex Taq kit (TaKaRa). The primers used for ADO gene were 5' -GGAGCACTGTTTCTCCCTTTT-3' and 5' -CAATCAAGAGGGCTTAGACGA-3' . The primers used for Wnt5a gene were 5' -GTGCAATGTCTTCCAAGTTCTTC-3' and 5' -GGCACAGTTTCTTCTGTCCTTG-3' . The expression of β-actin was used for normalization with primers of 5' -CGAGGCCCAGAGCAAGAGAG-3' and 5' -CTCGTAGATGGGCACAGTGTG-3' . The relative expression was calculated using the algorithm of 2^(-ΔΔCt). Every gene's expression was evaluated at least using three biological replicates.

Methylation-specific PCR(MSP)

MSP reaction was carried out as described elsewhere (Jiang *et al.*, 2017). Briefly, genome DNA was isolated using DNAiso from TaKaRa. Part of the isolated DNA was treated with sodium bisulfite. The amplification reaction was performed using TaKaRa Ex Taq kit (TaKaRa) according to the instructions. The primers for methylation sites were 5'-GTATTTTTCGGAGAAAAAGTTATGC-3' and 5'-AACCGCGAATTAATATAAACGTC-3'. The unmethylated sites primers were 5'-GGTATTTTTTGGAGAAAAAGTTATGTG-3' and 5'-CAACCACAAATTAATATAAACATC-3'. The amplification products were resolved by 1% agarose gel electrophoresis.

Gene expression profile analysis

RNA sequencing gene expression analysis (RNAseq) was performed by Novogene (Beijing, China). The ΔADO and its control counterpart (Vct) cells were cultured using DMEM high glucose media. For gene expression analysis, three independent biological replicates of each cell line were included.

Western blot analysis

Total protein was isolated using RIPA Lysis and Extraction buffer from Thermo Fisher Scientific (Waltham, MA). Protein electrophoresis were conducted using Mini-PROTEAN TGX SDS-PAGE gels purchased from Bio-Rad (Hercules, CA). The mouse anti human Wnt5a, β-actin and goat anti mouse antibodies were purchased from Abcam (Cambridge, UK).

Hypotaurine quantification

The quantitation of intracellular hypotaurine was carried out as what had been described elsewhere (Gao *et al.*, 2016). Briefly, cells were harvested when they reached to 70-80% confluence. The cells were firstly washed using cold PBS for triplicates and then subjected to metabolite extraction using 1ml of 21:79 methanol/water and 2ml chloroform. After centrifugation, the supernatant of each sample was allocated to a new tube and lyophilyzed at −50°C. The AccQTag Ultra

Derivatization Kit from Waters (Milford, MA) was used for hypotaurine derivatization and the quantitation was carried out using 1290 Infinity ultra performance liquid chromatography system hyphenated to 6460 Triple Quad mass spectrometry system from Agilent. Each cell line was analyzed in triplicates biologically and the ion intensity was used for relative quantitation after calibrated against individual protein dry weight.

Results

ADO gene knockdown increased the expression of WNT5A

In order to confirm the ADO gene was properly knocked down, RT-PCR analysis was carried out to verify the expression of ADO gene. It was found that ΔADO U251 cells showed lower ADO gene expression (Fig. 1A). The ΔADO also exhibited lower intercellular hypotaurine level (Fig. 1B). RNAseq expression analysis of the ΔADO and its empty vector cell line (Vct) indicated that 96 genes were up-regulated and 218 genes were down-regulated owning to the ADO gene knockdown (Tab. S1). Pathway enrichment analysis based on the gene expression data showed that the mostly perturbated cellular process was the extracellular matrix (ECM)-receptor interaction process (Fig. 1C). In the genes involved in ECM-receptor interaction process, one of the mostly differentially expressed was Wnt5a (Fig. 2, p=0.0001).

Decreased WNT5A expression was caused by increased hypotaurine

Cysteamine can be utilized to synthesize hypotaurine via ADO pathway (Gao *et al.*, 2016). ΔADO and Vct U251 cells were cultured under the presence of 400 μM cysteamine or not. The invasion ability of ΔADO cells was not significantly affected by extra cysteamine (Fig. 3A).

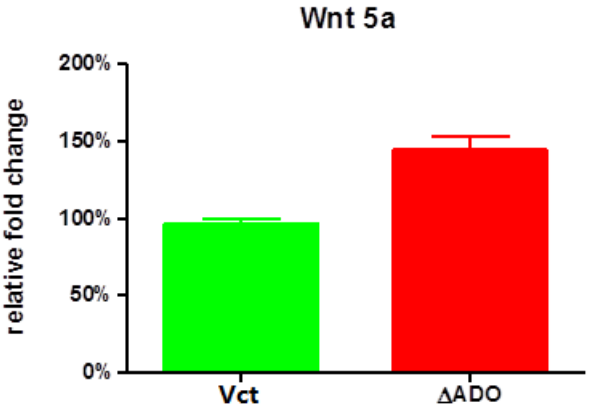

FIGURE 2. The expression of Wnt5a in the genetically modified U251 cell lines.

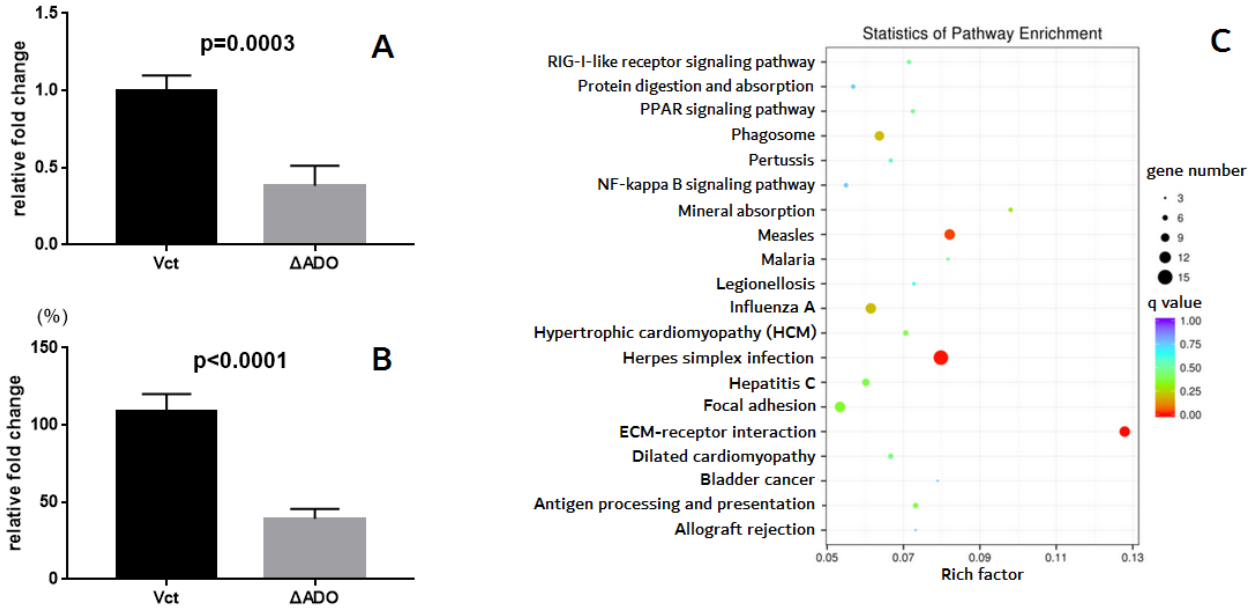

FIGURE 1. Effects of ADO gene knockdown on the U251 cells. (A) Relative ADO gene expression analysis by RT-PCR. The ΔADO data were normalized against that of the Vct. (B) Intercellular hypotaurine content comparison between the two cells. The ΔADO data were normalized against that of the Vct. (C) Gene expression enrichment analysis revealed the most significantly perturbated pathways due to ADO gene knockdown. The sizes of the dots represented the perturbated genes in the corresponding pathways. The color of the dots indicated the rich factors, which were reversely correlated with the q values. The bigger the q value, the less the statistical significance.

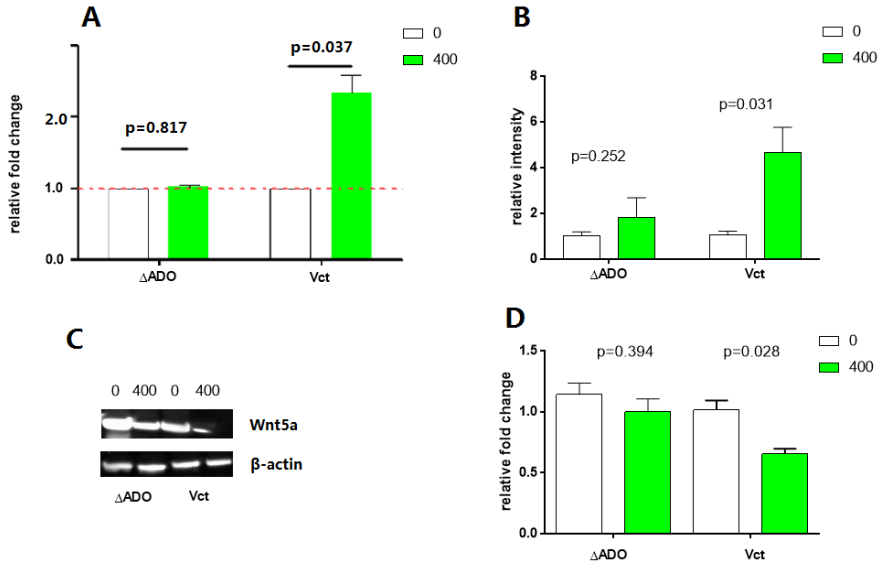

FIGURE 3. Modulating intercellular hypotaurine affected Wnt5a expression and the invasion abilities of the ADO gene genetically modified cells. (A) ΔADO and Vct cells were cultured with 400 μM cysteamine or not individually. Cysteamine only increased the invasion ability of Vct cells. (B) Intercellular hypotaurine increased in Vct cells when they were cultured at the presence of 400 μM cysteamine. (C) Western blot results of the WNT5a protein expression of the two cell lines challenged by 400 μM cysteamine or not. WNT5a protein was decreased by cysteamine only in Vct Cells. This result could be verified by the RT-PCR analysis of the Wnt5a mRNA expression (D).

When Vct cells were challenged by cysteamine, the invasion ability increased evidently (Fig. 3A). Notably, when the two cell lines were stimulated by cysteamine intercellular hypotaurine increased significantly only in Vct cells (Fig. 3B). As of the Vct cells their increased intercellular hypotaurine was accompanied by decreased intercellular Wnt5a protein and compromised Wnt5a gene expression (Fig. 3C, D). These differential Wnt5a gene and protein expression features could not be found in cysteamine- stimulated ΔADO cells (Figs. 3C, D).

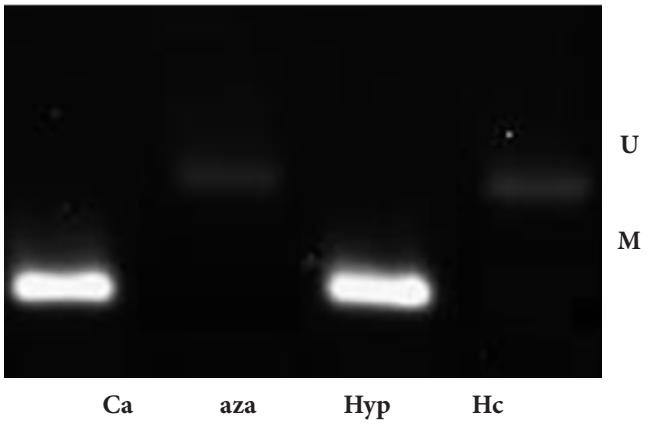

FIGURE 4. Wnt5a promoter region methylation status evaluation of U251 cells by methylation-specific PCR analysis. The PCR was performed for each treatment using the two pairs of primers individually. For each treatment, the two PCR products were mixed together to be separated by gel electrophoresis. Ca: DMSO control; Aza: 5-aza-2'-deoxycytidine treated cells; Hyp: 10μM hypotaurine treated; Hc: PBS treated control. U: unmethylated; M: methylated.

Hypotaurine increased methylation status of Wnt5a promoter

ΔADO U251 cells were treated with 5-aza-2›-deoxycytidine for 3 days. The Wnt5a promoter region methylation status was evaluated using methylation-specific PCR as described previously (Jiang *et al.*, 2017). It was found that the untreated cells showed hypermethylation status of Wnt5a promoter region than that of the treated cells (Fig. 4). When the ΔADO cells were treated with hypotaurine, their Wnt5a promoter methylation levels were also higher than that of the untreated ones (Fig. 4). This proved that hypotaurine could enhance the methylation status of Wnt5a promoter.

Discussion

The notorious nature of gliomas, especially the higher grades gliomas, is their invasive and infiltrative growth. Glioma cells do not migrate through intravascular or lymphatic routes as what are usually adopted by other solid tumors (Beauchesne, 2011). Invasion of gliomas was achieved by cells moving through extracellular space of the adjacent normal brain tissue. To finish this movement, glioma cells must degrade or adhere to the ECM firstly (Bolteus *et al.*, 2001; Cuddapah *et al.*, 2014). ADO gene deficiency had been demonstrated to decrease hypotaurine synthesis and compromise the invasion ability of U251 cells (Gao *et al.*, 2016). From the RNAseq analysis, we noticed that hypotaurine deficiency significantly affect the ECM-receptor pathway (Fig. 1). This indicated that hypotaurine was closely related to the invasion ability of glioma cells.

Wnt5a is one of the members of Wnt pathway glycoproteins. It involves in multiple developmental and oncogenesis pathways (McDonald and Silver, 2009). That it acts as a tumor promoter or suppressor is still elusive. In a neuroblastoma study, Blanc *et al.* (2005) found that metastatic neuroblasts were characterized by downregulated Wnt5a expression compared to the primary tumors. Ying *et al.* (2008), analyzed normal colon, colorectal cancer and patient tumor cells and proved that Wnt5a was frequently inactivated in malignant colon cells. The lower expression of Wnt5a was due to its promoter's hypermethylation (Ying *et al.*, 2008). These results indicated that Wnt5a seemed to be a tumor suppressor. On the other hand, the fact that Wnt5a was a tumor promoter was also evident. For example, in a

non-small-cell lung carcinoma analysis, it was found that when Wnt5a was suppressed by microRNA, the proliferation, migration, and invasion ability of the malignant cells were impaired (Liu *et al.*, 2018). The similar evidence could also be found in a leukemia cell analysis, which showed that Wnt5a facilitated migration and invasion through PI3K/Akt-RhoA pathway (Deng *et al.*, 2017).

In this assay, Wnt5a was identified as one of the most significantly perturbated genes in ECM-receptor pathway (Fig. 2). Lower intracellular hypotaurine facilitated the expression of Wnt5a. *In vitro* analysis demonstrated that applying hypotaurine-synthesis substrate cysteamine increased intracellular hypotaurine and enhanced cell invasion ability (Figs. 3A, B). Decreased intracellular Wnt5a proteins (Fig. 3C) accompanied this phenotype. Besides the protein assay, the transcription level evidence also proved the decreased Wnt5a mRNA expression in higher intracellular hypotaurine content condition (Fig. 3D). Because hypotaurine synthesis deficient cells were less invasive, Wnt5a seemed to be a tumor suppressor.

Hypotaurine could inhibit demethylases. Epigenetic modification of Wnt5a promoter plays key roles in oncogenesis (Gao *et al.*, 2017; Jiang *et al.*, 2017). In this light, we hypothesized that promoter methylation might be one of the possible mechanism, through which hypotaurine attenuated Wnt5a's expression. In order to test the hypothesis, a demethylation agent 5-aza-2'-deoxycytidine was selected as control. 5-aza-2'-deoxycytidine can antagonize the methylation process as exhibited in Fig. 4. As expected, the promoter methylation status was enhanced in hypotaurine treated ΔADO cells (Fig. 4).

As what had been described previously, the exact effects of Wnt5a on oncogenesis was elusive. Many studies aiming at gliomas tended to regard Wnt5a as an invasiveness promoter (Binda *et al.*, 2017; Hu *et al.*, 2016). The discrepancy between previous reports and this study might be that this study was conducted only using a genetically modified cell line. Whether ADO gene itself also involved in pathways beyond hypotaurine synthesis was still unknown. Rationally, this conclusion should be verified using real glioma samples.

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