

RESEARCH ARTICLE

Expression Profile Analysis of Zinc Transporters (ZIP4, ZIP9, ZIP11, ZnT9) in Gliomas and their Correlation with IDH1 Mutation Status

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Abstract

Background: Zinc transporters have been considered as essential regulators in many cancers; however, their mechanisms remain unknown, especially in gliomas. Isocitrate dehydrogenase 1 (IDH1) mutation is crucial to glioma. This study aimed to investigate whether zinc transporters are correlated with glioma grade and IDH1 mutation status. **Materials and Methods:** IDH1 mutation status and mRNA expression of four zinc transporters (ZIP4, ZIP9, ZIP11, and ZnT9) were determined by subjecting a panel of 74 glioma tissue samples to quantitative real-time PCR and pyrosequencing. The correlations between the expression levels of these zinc transporter genes and the grade of glioma, as well as IDH1 mutation status, were investigated. **Results:** Among the four zinc transporter genes, high ZIP4 expression and low ZIP11 expression were significantly associated with higher grade (grades III and IV) tumors compared with lower grade (grades I and II) counterparts ($p < 0.0001$). However, only ZIP11 exhibited weak correlation with IDH1 mutation status ($p = 0.045$). Samples with mutations in IDH1 displayed higher ZIP11 expression than those without IDH1 mutations. **Conclusions:** This finding indicated that zinc transporters may interact with IDH1 mutation by direct modulation or action in some shared pathways or genes to promote the development of glioma. Zinc transporters may play an important role in glioma. ZIP4 and ZIP11 are promising molecular diagnostic markers and novel therapeutic targets. Nevertheless, the detailed biological function of zinc transporters and the mechanism of the potential interaction between ZIP11 and IDH1 mutation in gliomagenesis should be further investigated.

Keywords: Glioma-isocitrate dehydrogenase 1 (IDH1) - zinc transporter-expression

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Introduction

Gliomas are among the most common and almost incurable tumors of central nervous system neoplasms. The management of glioma patients has undergone great changes over the past 20 years and understanding of glioma on molecular level has greatly expanded. Identification of tumor-specific biomarkers has played an important role in the diagnosis, prognosis, and tailored treatment of cancers, thereby improving the survival and quality of life for patients (Cancer Genome Atlas Research Network, 2008; Bleeker et al., 2012).

Zinc plays vital roles in many biological processes in the human body; the expression of zinc transporters in the cellular membrane is essential to maintain zinc homeostasis and normal biological activities (Lichten and Cousins, 2009a; Maret, 2013). Two types of transporters have been identified, the ZIP (encoded by SLC39) family and the ZnT (encoded by SLC30) family, which act as zinc importers and zinc exporters in cellular zinc

homeostasis, respectively. To date, 10 ZnTs and 14 ZIPs have been identified in mammals (Lichten and Cousins, 2009a; Lichten and Cousins, 2009b). Dysregulation of zinc and zinc transporters have been reported to have a relationship to several types of cancers, such as prostate, breast, and pancreatic cancers, and brain disorders, such as Alzheimer's disease, Parkinson's disease, and epilepsy (Michalczyk et al., 2002; Lonnerdal, 2007; Taylor et al., 2008; Costello et al., 2011; Chen et al., 2012; Franz et al., 2013; Kambe et al., 2014). A recent study has investigated the gene profiles of zinc transporters in patients with glioma, and found that several genes, especially ZIP4 up-regulation, are significantly associated with higher grade of gliomas and shorter overall survival. This finding indicates that ZIP4 may serve as a potential diagnostic and prognostic marker for gliomas. In addition, ZIP9, ZIP11, and ZnT9 were significantly associated with the grades and survival of glioma subjects in the Chinese cohort, but not validated in the cohort from the US (Lin et al., 2013). Only few studies have investigated the functional relevance of

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zinc transporters in glioma; the effect of zinc and zinc transporters on glioma tumorigenesis and aggressiveness remains unclear. However, zinc depletion causes increased oxidative stress and induces programmed cell death in many cells (Nardinocchi et al., 2010), whereas zinc hampers hypoxia stimulated hypoxia-inducible factor-1 (HIF-1) activation in astrocytes by inhibiting nuclear HIF-1 α translocation and heterodimerization (Kim et al., 2008). Therefore, zinc transporters may have a relationship with HIF activation and affect the development or progression of tumor cells.

IDH1 mutation as a new outstanding biomarker of glioma has been discovered by next-generation sequencing, mutations in IDH1 have been consistently found in codon 132 for arginine (R132) (Parsons et al., 2008). Mutation rarely occurs in primary glioblastomas (0.05%) but often occurs at higher frequencies in secondary glioblastoma (84.6%) and low-grade gliomas (70%-100%) (Balss et al., 2008; Ducray et al., 2009). These studies have demonstrated that IDH1 mutation is associated with improved survival in glioma patients, suggesting that IDH1 mutation could serve as reliable prognostic marker for low-grade glioma patients (Dubink et al., 2009; Krell et al., 2013) and may provide clinicians with a more comprehensive understanding of the IDH1 gene, especially IDH1 mutation event (Wang J-B et al., 2014). Although the exact mechanism by which IDH1 mutation leads to gliomagenesis is not fully understood, evidence shows that mutations in IDH1 are early events in gliomagenesis (Watanabe et al., 2009) and could induce genome-wide hypermethylation in isogenic tumor cells (Duncan et al., 2012; Turcan et al., 2012), leading to altered expression of a large number of genes (Masica and Karchin, 2011). It seems like a molecular switch to drive the occurrence of key tumor-related genes, thereby promoting glioma formation. One tested mechanism is that IDH1 mutation likely plays a part in oxidative stress response and alters hypoxic response. The reduced catalytic ability of IDH may lead to increased levels of HIF-1 α , which facilitates tumor growth (Zhao et al., 2009).

We assumed that IDH1 mutation may have certain interactions with zinc transporter genes in gliomagenesis, either by direct modulation of IDH1 mutation to the expression of zinc transporters, or through their influence on some shared pathways, such as hypoxic response, to induce tumor formation. Therefore, we selected four zinc transporter genes, namely, ZIP4, ZIP9, ZIP11, and ZnT9, which demonstrated significant association with glioma grades and survival in reported Chinese glioma patients

to test our hypothesis (Lin et al., 2013). We analyzed the expression profile of these zinc transporter genes in 74 glioma samples and further investigated their correlation with IDH1 mutation status.

Materials and Methods

Tumor specimens

A total of 74 fresh frozen glioma samples were collected from Tangdu Hospital of Fourth Military Medical University (Xi'an China). Tumor tissue samples were obtained by surgical resection before treatment with radiation and chemotherapy. Resected specimens were quick-frozen in liquid nitrogen and kept at -80°C. Tumors were reviewed by two independent neuropathologists to assign histological subtypes and grades according to the World Health Organization (WHO) criteria [1]. Clinical data were retrieved from the hospital patient records. This study was approved by the Ethics Committee of Tangdu Hospital; written informed consent was obtained from all patients.

mRNA expression determined by quantitative real-time reverse-transcription PCR (qRT-PCR)

Total RNA was extracted from glioma tissues using Trizol reagent (Qiagen, Hilden, Germany) following the instructions of the manufacturer. RNA was quantified using a Nanodrop spectrophotometer (Thermo scientific, Fitchburg, WI, USA). The mRNA expression level of four target genes (ZIP4, ZIP9, ZIP11, and ZnT9) and reference gene (β -actin) in the 74 samples was analyzed with TaqMan probe based one-step qRT-PCR using highly specific TaqMan probes and primers (Table 1). All experiments were performed with Applied Biosystems 7500 Real-Time PCR System (ABI, Carlsbad, CA, USA) using One Step PrimeScript™ RT-PCR Kit (TAKARA, Dalian, China). Briefly, reactions were carried out in 20 μ L reaction system, wherein each reaction contains 0.4 μ L of each Primer and Probe Mix (20 μ M), 2 μ L of total RNA (50 ng/ μ L), 10 μ L of 2 \times One Step RT-PCR Buffer III, 0.4 μ L TaKaRa Ex Taq HS (5 U/ μ L), and 0.4 μ L of PrimeScript RT enzyme Mix II. Thermo cycling conditions were set as the initial polymerase activation step for 10s at 95°C, followed by 40 cycles of 5 s at 95°C for template denaturation, 34s at 60°C for annealing, extension, and fluorescence detection. All samples were amplified in technical triplicates. Negative controls without template were included in each run. Both agarose electrophoresis profile of the qRT-PCR products and dissociation curve analysis were used to check the specificity of qRT-PCR.

Table 1. Primers and Probes Used in the Present Study

Genes	Primers	Probes	Amplicon size
ZIP4 (SLC39A4)	F:5'-CGAGGTCCCTATGACGCTG-3' R:5'-TGTTGCTGGAGCTGATGAGGG-3'	5'-FAM-AGGGTCCCGGCTGCTGGCT-BHQ2-3'	181bp
ZIP9 (SLC39A9)	F:5'-AGGGCAGAGACAGCTTTGTG-3' R:5'-CGAGCCTGGGATGAGGAAAG-3'	5'-FAM-TTGCTGCTGAACCCCTAGGACCCAT-BHQ2-3'	92bp
ZIP11 (SLC39A11)	F:5'-GACCTCCTGATGCCTCACT-3' R:5'-TATCCGGATGGAAAGTTCCT-3'	5'-FAM-AGCAGAAGACCCAGACGACCCT-BHQ2-3'	144bp
ZnT9 (SLC30A9)	F:5'-GCCTGTAATCCAGCGACC-3' R:5'-ATGATGGTACTTTTTCCACTCTGA-3'	5'-FAM-TGTGATCCCTGTCTTCTTTCTGAACAT-BHQ2-3'	181bp

Table 2. Clinical Characteristics of the 74 Glioma Patients That Participated in This Study

Variables		No. (%)
Age (years)	Median	46
	Range	10-77
Sex	Female	32(43.1%)
	Male	42(56.9%)
WHO grades	Grade I	3(4.1%)
	Grade II	38(51.4%)
	Grade III	23(32.4%)
	Grade IV	10(12.2%)
Histologic subtype	Astrocytoma	27 (36.5%)
	Anaplastic astrocytoma	23 (31.0%)
	Oligodendroglioma	11 (14.8%)
	Glioblastoma	9 (12.2%)
	Gliocytoma	3 (4.1%)
	Meduloblastoma	1 (1.4%)
Total		74

To determine the gene expression present in the tested samples, the average threshold cycle (Ct) values for the target and reference genes were obtained from each reaction.

Before quantification of mRNA expression in the tumor samples, standard curves were established to determine the amplification efficiency of each gene using serial dilutions of total RNAs (50, 10, 2, 0.4, and 0.08 ng/ μ L) from one of the 74 tumor samples. PCR reaction efficiency (E) was estimated using the following formula: $E (\%) = (10^{[1/\text{slope}] - 1}) \times 100$. The relative mRNA expression level of each targeted gene was normalized against selected reference genes, as calculated by the $E^{-\Delta Ct}$ method, where $\Delta Ct = Ct_{\text{targeted gene}} - Ct_{\text{reference gene}}$.

IDH1 mutation detection by pyrosequencing

Genomic DNA from 50 glioma tissue samples was isolated using E.Z.N.A.TM Tissue DNA Kit (OMEGA Bio-Tek, USA) according to the instructions of the manufacturer. The IDH1 mutation status in the 50 samples were examined by pyrosequencing. Exon 4 of IDH1 containing the R132 coding region was amplified using the following primers: Fp-5'-CACCATACGAAATATTCTCG-3', Rp-5'-biotin-CAACATGACTTACTTGATCC-3'. Afterward, 10 μ L of the PCR product and the control were subjected to pyrosequencing on a PyroMark Q24 System with the Pyro Gold Reagent Kit (both by Qiagen) using the sequencing primer 5'-GTGAGTGGATGGATGGGTAAACC-3'. Subsequent purification and processing of the biotinylated single-strand DNA was performed according to the instructions of the manufacturer. Resulting data were analyzed and quantified with PyroMark Q24 Software (Qiagen). All the primers and the detailed experimental procedure were described previously (Setty et al., 2010).

Statistical analysis

Statistical analyses were conducted with GraphPad Prism software (Version 4.0, San Diego, CA, USA). Mann-Whitney U test was used to analyze the associations between the expression level of the four genes (ZIP4, ZIP9, ZIP11, and ZnT9) and pathological grades or IDH1 mutation status. All statistical tests were 2-sided.

Differences with $p < 0.05$ were considered statistically significant, unless otherwise indicated.

Results

Patient characteristics

The characteristics of the 74 gliomas are listed in Table 2. The sex ratio of these samples was 1.31 (42 men and 32 women), and median age was 46 years (range, 10 to 77 years). These gliomas consisted of 3 WHO grade I (4.1%), 38 WHO grade II (51.4%), 23 WHO grade III (32.4%), and 10 WHO grade IV gliomas (12.2%). With respect to histological subtype, 27 astrocytomas (36.5%), 23 anaplastic astrocytomas (31%), 11 oligodendrogliomas (14.8%), 9 glioblastomas (12.2%), 3 gliocytomas (4.1%), and 1 meduloblastoma (1.4%) were obtained. Among the 9 glioblastomas, 4 were primary tumors, whereas 5 were secondary tumors.

IDH1 mutation status in gliomas

We sequenced 50 of the 74 tumors with available DNA for IDH1 mutations. In total, 26 mutations (52%) in IDH1 codon 132 were detected; all mutations were

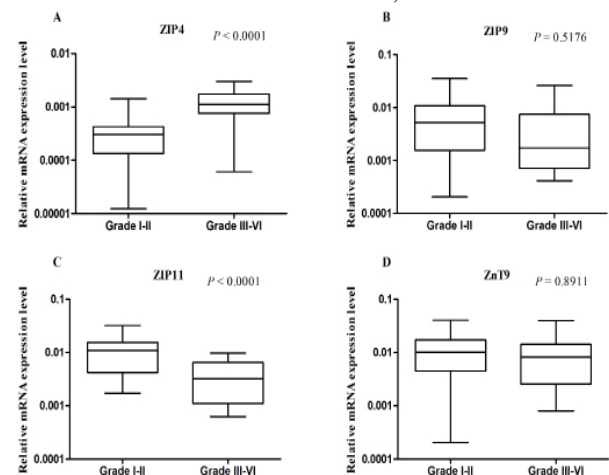


Figure 1. Correlation between mRNA Expression Levels of ZIP4, ZIP9, ZIP11, and ZnT9 and Glioma Grades

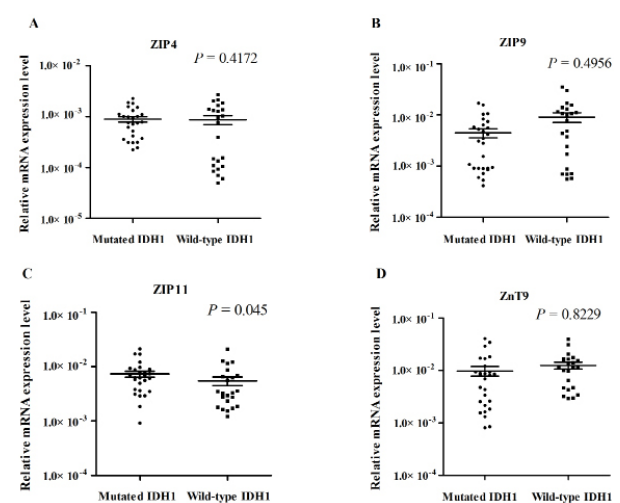


Figure 2. Association of mRNA Expression Levels of ZIP4, ZIP9, ZIP11, and ZnT9 with IDH1 Mutation Status in Glioma Samples

Table 3. IDH1 Mutation Frequency According to Glioma Grades and Histological Subtypes

Histologic subtype	Glioma grades (N)	IDH1 mutation status		Mutation frequency
		Wild-type, N	Mutated, N	
Gliocytoma	Grade I (1)	1	0	0
Oligodendrocytes astrocytoma	Grade II (6)	1	583.3	0%
Astrocytoma	Grade II (16)	8	8	50.00%
Gliocytoma	Grade II	1	1	50.00%
Anaplastic astrocytoma	Grade III (19)	10	9	47.30%
Primary glioblastoma	Grade IV (2)	2	0	0
Secondary glioblastoma	Grade IV (4)	1	3	75%
Total		24	26	52%

R132H type and heterozygous with one wild type allele being present. The prevalence of mutations in tumors with different subtypes is shown in Table 3. IDH1 mutations were more frequent in grade II tumors than grades III or IV tumors, affecting 14 (58.3%) of the 24 grade II, 9 (47.3%) of the 19 grade III, and 3 (50%) of the 6 grade IV gliomas. Moreover, IDH1 mutation was more prevalent in oligodendrocytes astrocytoma of grade II and secondary glioblastomas, with a frequency of 83.3% and 75%, respectively.

Expression profile of ZIP4, ZIP9, ZIP11, and ZnT9 in gliomas

According to the standard curve analysis, the amplification efficiency of three target genes and one reference gene (ACTB) was approximately 1.9; thus, the mRNA expression level of each sample was quantified with the $1.9^{-\Delta\Delta Ct}$ method. The relative mRNA expression value of ZIP4, ZIP9, ZIP11, and ZnT9 in the 74 tumor samples is summarized in Table 4. A huge variability in the expression level of the zinc transporter genes was observed in the studied samples. The variation fold of these four genes ranged from 51.6 to 242.5; ZIP11 showed the smallest variation fold, whereas ZIP4 presented the largest variation fold. Considering the different grades of gliomas, these four genes showed the largest variation fold in grade II samples than samples with grades I, III, or IV. We performed an association analysis between the gene expression level and glioma grades (Figure 1), and found that ZIP4 was positively correlated with glioma grades, with significantly higher expression level in higher grades (grades III-IV) than in lower grades (grade I-II) of glioma samples ($p < 0.001$). By contrast, ZIP11 was negatively correlated with glioma grades, having significantly higher expression level in lower grades (grades I-II) than in

higher grades (grades III-IV) of glioma samples ($p < 0.001$). ZIP9 and ZnT9 showed the same trend as ZIP11, but the difference did not reach statistically significant level ($p = 0.5176$ and $p = 0.8911$, respectively).

Correlation between mRNA expression level and genotype of IDH1 in glioma

We further analyzed the association between IDH1 genotype and mRNA expression levels of the four transporter genes based on the Mann-Whitney U test (Figure 2). The results showed that only the expression level of ZIP11 was weakly correlated to the IDH1 mutation status of the studied samples, with higher expression level in IDH1 mutated samples than in IDH1 wild-type ones ($p = 0.045$). The other three genes did not show any correlation with IDH1 genotype ($p > 0.05$).

Discussion

We investigated the expression profile of four zinc transporter genes in glioma samples and explored their correlation with IDH1 mutation status for the first time. We found that ZIP4 and ZIP11 were correlated with tumor grade; only ZIP11 had weak correlation with IDH1 mutation. These results indicated that ZIP4 and ZIP11 could be used as potential diagnostic biomarkers of glioma. ZIP11 may have certain interaction with mutations in IDH1 that contributed to formation and progression of glioma.

Zinc and zinc transporters are critical in maintaining the normal biological activities of humans (Maret, 2013; Kambe et al., 2014). In recent years, more attention has been paid to the functional relevance of zinc transporters to cancer pathogenesis and abnormal expression of zinc importers have been identified in many cancer types, including prostate, breast, and pancreatic cancers (Michalczyk et al., 2002; Lonnerdal, 2007; Taylor et al., 2008; Costello et al., 2011; Chen et al., 2012; Franz et al., 2013; Kambe et al., 2014). However, similar studies performed in gliomas are very few. A newly published study has investigated the gene profile of 24 zinc transporter genes in patients with glioma (Lin et al., 2013). ZIP4 was significantly associated with tumor grades and overall survival in three independent cohorts, one Chinese cohort and two US cohorts. High ZIP4 expression was significantly associated with higher grade of gliomas and shorter overall survival. In addition, ZIP9, ZIP11, and ZnT9 also exhibited significant effect in the Chinese

Table 4. mRNA Expression Levels of ZIP4, ZIP9, ZIP11, and ZnT9 According to Glioma Grades

Genes	Values	Glioma grades (n)				
		I (3)	II (38)	III (23)	IV (10)	Total(74)
ZIP4	Max/min(fold)	3.64E-03/7.19E-04 (5.1)	1.17E-02/1.04E-04 (112.5)	2.52E-02/1.80E-03 (14.2)	1.92E-02/5.08E-04 (37.7)	2.52E-02/1.04E-04 (242.5)
	Median	3.01E-03	2.36E-03	9.35E-03	1.15E-02	3.89E-03
	Mean ±SD	2.46E-03±1.54E-03	2.79E-03±2.31E-03	1.05E-02±5.80E-03	1.19E-02±5.80E-03	6.43E-03±5.82E-03
ZIP9	Max/min(fold)	1.40E-02/3.57E-04 (39.2)	3.54E-02/2.05E-04 (172.9)	3.62E-02/4.16E-04 (62.9)	1.45E-02/3.57E-04 (19.2)	3.54E-02/2.05E-04 (172.9)
	Median	5.19E-03	5.41E-03	9.10E-04	6.41E-03	4.61E-03
	Mean ±SD	6.52E-03±6.93E-03	7.68E-03±7.89E-03	4.03E-03±6.28E-03	7.31E-03±4.40E-03	6.40E-03±7.08E-03
ZIP11	Max/min(fold)	1.68E-02/8.74E-03 (1.9)	3.22E-02/1.72E-03 (18.7)	9.68E-03/6.24E-04 (15.5)	6.63E-03/7.36E-04 (9.0)	3.22E-02/6.24E-04 (51.6)
	Median	1.61E-02	1.08E-02	3.60E-03	1.81E-03	6.29E-03
	Mean ±SD	1.39E-02±4.47E-03	1.05E-02±6.76E-03	4.37E-03±2.91E-03	2.70E-03±2.21E-03	7.71E-03±6.28E-03
ZnT9	Max/min	2.36E-02/3.57E-04 (66.2)	4.04E-02/2.05E-04 (196.9)	3.11E-02/8.43E-04 (36.9)	3.94E-02/8.05E-04 (49.0)	4.04E-02/2.05E-04 (197.3)
	Median	2.06E-02	1.00E-02	8.30E-03	4.30E-03	9.06E-03
	Mean±SD	1.49E-02±1.27E-02	1.19E-02±9.34E-03	9.58E-03±7.81E-03	9.88E-03±1.23E-02	1.10E-02±9.29E-03

cohort, but this result was not validated in the US cohort. Therefore, ZIP4 may serve as a potential diagnostic and prognostic marker for gliomas. To validate these findings, we selected four transporter genes (ZIP4, ZIP9, ZIP11, and ZnT9) to analyze their expression profile in 74 glioma tissues. We found that ZIP4 and ZIP11, but not ZIP9 and ZnT9, were significantly associated with tumor grade. High ZIP4 expression and low ZIP11 expression were correlated with higher grade of gliomas, consistent with the previous findings. ZIP4 is one of the most studied zinc transporters in maintaining zinc homeostasis in humans. ZIP4 overexpression was also linked to enhanced tumorigenesis and progression in pancreatic cancer (Li et al., 2007). However, ZIP4 expression was down-regulated and exhibited an inhibitory effect on cell proliferation and invasion in prostate cancer (Chen et al., 2012). Therefore, the exact function of zinc transporters may be cancer-type specific (Lin et al., 2013). In addition, both as zinc importers, ZIP11 showed negative correlation with glioma grade. Abundant expression of ZIP11 was identified in the stomach, cecum, and colon tissues of mouse (Martin et al., 2013), but its expression in tumors was hardly reported. The down-regulation of other zinc importers, such as ZIP3 and ZIP14, has been reported to be likely involved in progression of pancreatic cancer (Costello and Franklin, 2013) and hepatic cancer (Franklin et al., 2012). These findings also indicated that the biological roles of zinc transporter genes were complicated and may present different effects in different physiologic conditions.

To further expand our understanding on the molecular biology of glioma pathogenesis, we performed a correlation analysis between the expression level of zinc transporter genes and IDH1 mutation status of gliomas. IDH1 mutations have become an increasingly important topic, both in patients with brain tumors and non-brain malignancies. The mutation status of IDH1 in 50 glioma samples showed that IDH1 mutation was more prevalent in grade II and III gliomas and secondary glioblastomas, with occurrence of more than 50%. This finding was in agreement with previous reports (Balss et al., 2008; Ducray et al., 2009). The correlation analysis demonstrated that ZIP11 expression was significantly associated with the IDH1 mutation status of glioma samples. The samples with IDH1 mutations exhibited higher ZIP11 expression level than those without IDH1 mutations. This result suggested that a direct or indirect interaction may exist between ZIP11 expression and IDH1 mutations in the whole network of glioma formation. As IDH1 mutations are proved to be early molecular events in the development of glioma and are correlated with the alteration of a large number of genes, including oncogenes, suppressor genes, and survival genes. Therefore, IDH1 mutations may directly modulate ZIP11 expression in glioma cells. In addition, considering that both IDH1 mutation and zinc transporters take part in oxidative stress response and related to the HIF expression (Kim et al., 2008; Zhao et al., 2009), they may act on the same pathway or cancer-related gene to promote tumor growth. However, further research is necessary to testify these hypotheses.

Our study has several limitations. First, the sample size is insufficient to analyze the relationship between

expression level of zinc transporters and the specific pathological subtype. Second, limited tissue also hampered us to determine the protein expression level of the tested genes. In addition, lack of prognosis information on the samples limited us in analyzing the correlation between expression profile and patient survival. Therefore, to confirm and extend our findings, additional large-scale studies using glioma samples well-characterized with respect to external factors are needed.

In conclusion, the findings presented in this study demonstrated that the aberrant expression of zinc transporters is essential to glioma and different members may have a special role in gliomagenesis. In addition, ZIP11 expression may have certain interaction with mutations in IDH1 to contribute to the development of glioma. Further studies focused on zinc transporters and zinc-regulated biological functions may provide deep insights into the basis of glioma pathogenesis and will offer promising biomarkers to develop new targeted therapies for glioma.

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