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ZIP4 is a novel molecular marker for glioma

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Background. Dysregulated zinc transport has been observed in many cancers. However, the status of zinc homeostasis and the expression profile of zinc transporters in brain and brain tumors have not been reported.

Methods. The gene profiles of 14 zinc importers (ZIPs) and 10 zinc exporters (ZnTs) in patients with glioma were studied by investigating the association between the zinc transporters and brain tumor characteristics (tumor grade and overall survival time). Three independent cohorts were analyzed to cross-validate the findings: the Chinese Glioma Genome Atlas (CGCA) cohort ($n = 186$), the US National Cancer Institute Repository for Molecular Brain Neoplasia Data (REMBRANDT) cohort ($n = 335$), and The University of Texas (UT) cohort ($n = 34$).

Results. The expression of ZIP3, 4, 8, 14, ZnT5, 6, and 7 were increased, and the expression of ZnT10 was decreased in grade IV gliomas, compared with grade II gliomas. Among all 24 zinc transporters, ZIP4 is most significantly associated with tumor grade and overall survival; this finding is consistent across 2 independent cohorts (CGCA and REMBRANDT) and is partially validated by the third cohort (UT). High ZIP4 expression was significantly associated with higher grade of gliomas and shorter overall survival (hazard ratio = 1.61, 95% confidence interval = 1.02–2.53, $P = .040$ in CGCA cohort; hazard ratio = 1.32, 95% confidence interval = 1.08–1.61, $P = .007$ in REMBRANDT cohort).

Conclusions. Dysregulated expression of zinc transporters is involved in the progression of gliomas. Our results suggest that ZIP4 may serve as a potential diagnostic and prognostic marker for gliomas.

Keywords: biomarker, brain tumor, prognosis, survival, zinc transporter, ZIP4.

Zinc is an essential trace element in human body and a cofactor for many enzymes.[1] Zinc-binding proteins account for more than half of the transcription regulatory proteins in human body.[2] Zinc plays a critical role in many basic biological processes, such as the metabolism of nucleic acids, proteins, carbohydrates, and lipids, as well as the regulation of gene transcription, cell growth, development, and differentiation.[3] Zinc deficiency often leads to growth retardation, reduced food intake, impaired immune activity, and diminished brain function.[4] As part of the catalytic center of over 300 metalloenzymes, such as DNA and RNA polymerases, carbonic anhydrase, and matrix metalloproteinase (MMP), zinc is highly involved in hypoxia, angiogenesis, cell proliferation, and metastasis of cancer. Zinc depletion causes increased oxidative stress and induces programmed cell death in many cells. However, excessive zinc can also be cytotoxic.[5] Therefore, a complex zinc transport network has evolved to maintain the balance of zinc uptake, intracellular storage, and efflux.[6]

Zinc is the second most abundant trace element in brain after iron.[7,8] Maternal zinc deficiency has been linked to congenital malformations of the central nervous system.[9] Impaired brain development due to zinc deficiency was also observed in rats.[10,11] Because it is critical to maintain a redox state, the concentration of zinc in the brain is tightly

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regulated.[12–15] In the SPECT imaging performed in rats, it has been shown that zinc uptake in glioma xenografts was 3–10 times higher than that in the normal brain.[16] Zinc cannot diffuse freely through the cell membrane; therefore, it uses membrane proteins as a way to cross membrane to maintain zinc homeostasis and keep a balance between apoptosis and cell growth. Two solute-linked carrier (*SLC*) gene families were identified in zinc transport, the ZIP (ZRT, IRT-like protein, encoded by *SLC39*) family and ZnT (Zinc Transporter, encoded by *SLC30*) family. They appear to have opposite roles in cellular zinc homeostasis. ZIP transporters increase intracellular zinc concentration by transporting zinc into cytosol either from extracellular space or from the lumen of intracellular compartments. Conversely, ZnTs efflux zinc ions and store excessive zinc in intracellular compartments.[17] Aberrant zinc transporter expression has been observed in several cancers, such as breast and pancreatic cancer.[18–20]

Many zinc transporters are expressed in brain tissues.[21] Alterations of zinc and zinc transporters have been reported in certain pathological conditions, such as Alzheimer's disease,[22] Parkinson's disease,[23] and epilepsy.[24] Many of these studies focus on the zinc expression in neurons; however, glial cells that typically give rise to primary intrinsic brain tumors seem to react differently to changes of these elements.[25] Abnormal activation of intracellular pathways has different effects on neurons, and glial cells in brain may be explained by the fact that neurons are terminally differentiated cells and astrocytes retain the ability to self-renew and proliferate, which could potentially lead to either cancer or neurodegenerative disorders, depending on the cells affected.[26]

Diffuse astrocytomas are the most common glioma, and are often resistant to standard treatment, such as radiation or chemotherapy. The median survival times for grade II and grade III gliomas are 5–8 years and 2–3 years, respectively.[27] Grade IV astrocytomas (GBM) represent the most frequent and malignant form of astrocytomas, with a median survival time of only 14.6 months and a two-year survival rate of 5%–10%.[28] The mechanisms underlying gliomagenesis remain largely unknown, and the limited choices of reliable biomarkers for early detection and the lack of curative treatment contribute to its poor prognosis. Because dysregulated zinc and zinc transporters are implicated in different types of cancers that show distinctive expression pattern of zinc transporters, we investigated whether altered expression profile of zinc transporters contributes to glioma pathogenesis and progression. In this study, we examined the expression profile of 24 zinc transporters (14 ZIPs and 10 ZnTs) in human glioma tissues from 3 independent cohorts of patients with glioma and studied their associations with tumor grade and patient overall survival time.

Materials and Methods

Tissue Samples

We included all 555 available samples from 3 independent human glioma cohorts: Chinese Glioma Genome Atlas (CGCA) cohort ($n = 186$), the US National Cancer Institute Repository for Molecular Brain Neoplasia Data (REMBRANDT) cohort ($n = 335$), and the University of Texas (UT) cohort ($n = 34$). For the CGCA cohort, 186 frozen glioma tumor samples were obtained from patients with newly diagnosed glioma treated at the Glioma Center of Beijing Tiantan Hospital from 2006 through 2009. Clinicopathological information (age, sex, preoperational Karnofsky performance scale [KPS] score, and treatment) were obtained from medical records of Tiantan hospital. Tumor histology of all patients was confirmed independently by 2 neuropathologists on the basis of the 2007 edition of World Health Organization (WHO) classification of central nervous system tumors. All the patients in the study received conventional therapy consisting of maximal surgical resection, followed by radiotherapy and/or chemotherapy. For low-grade gliomas, the patients first underwent maximal resection and then received radiotherapy of 50–54 Gy in 1.8 Gy fractions. For high-grade gliomas, the patients received operation, followed by 60 Gy radiotherapy for 6 weeks with concurrent temozolomide chemotherapy, and then adjuvant temozolomide-based chemotherapy. Patients who received radiotherapy or chemotherapy before admission were excluded from this study. The study was approved by the Research Ethics Committee of Tiantan Hospital. Informed consent forms were obtained from all patients.

The second independent cohort is the REMBRANDT cohort. REMBRANDT is a joint program of the National Cancer Institute and the National Institute of Neurological Disorders and Stroke, which hosts clinical and genomics data from clinical trials involving patients with gliomas. Clinicopathological information of 335 patients with gliomas is available. In the third cohort, 34 surgically resected glioma tissue samples were obtained from the University of Texas Medical School at Houston (UT cohort). Clinical information of those de-identified samples was obtained through the database of the Neurosciences Research Repository at UT Medical School. This study was approved by the UT Institutional Committee for the Protection of Human Subjects.

Tumor grade is characterized as grade II (astrocytomas) and grade IV (glioblastomas). Because of limited availability of grade III glioma samples from the CGCA and UT cohorts and extreme difficulty to obtain normal brain tissue samples, we only included grade II and grade IV samples in this study. The overall survival time is defined as the interval from diagnosis to either death or the last observation taken. Data were censored at the last follow-up for living patients. Summary statistics of the CGCA and REMBRANDT cohorts are presented in Table 1.

RNA Extraction and Microarray Analysis

For the CGCA cohort, all the tissue samples were immediately snap-frozen in liquid nitrogen after surgery. A hematoxylin and eosin–stained frozen section was prepared for assessment of the percentage of tumor cells before RNA extraction. Only samples with greater than 80% tumor cells were selected. For microarray assay, total RNA

Table 1. Clinicopathological characteristics of the patients with glioma

Cohort	Diagnosis	Number of patients	Age (years)[a]	Sex (% female)	KPS score (range)	Median OS (95% CI) (months)	Number of deaths
CGCA	Grade II	98	38.5 ± 10.1	45	90 (70-100)	29.3 (NA)	8
	Grade IV	88	45.0 ± 12.9	41	80 (50-100)	13.8 (11.4-16.2)	62
REMBRANDT	Grade II	139	42.6 ± 14.2	33	90 (90-100)	42.6 (33.8-51.4)	139
	Grade IV	196	55.9 ± 12.6	36	90 (80-100)	16.8 (14.1-19.5)	196

[a]Mean ± standard deviation.
CI, confidence interval; KPS, Karnofsky performance status; OS, overall survival.

from frozen tumor tissues was extracted using the Total RNA Isolation Kit (Ambion, Austin, TX) according to the manufacturer's protocol. RNA concentrations were measured using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Houston, TX). The cDNA and biotinylated cRNA were synthesized and hybridized to the Agilent Whole Human Genome Array (Agilent, Santa Clara, CA) according to the manufacturer's instructions. Data were acquired using Agilent G2565BA Microarray Scanner System and Agilent Feature Extraction Software (version 9.1), and probe intensities were normalized using GeneSpring GX 11.0. A similar procedure was performed in the UT cohort to collect and process the tissues samples. All the samples were immediately snap-frozen in liquid nitrogen after surgery. Total RNA from frozen tumor tissue samples was extracted using the Total RNA Isolation Kit (Ambion, Austin, TX), and the concentration was measured using the NanoDrop ND-1000 spectrophotometer as above.

Real-Time Polymerase Chain Reaction (PCR)

The expression levels of ZIP4, matrix metallopeptidase 9 (MMP-9), vascular endothelial growth factor A (VEGF-A), platelet-derived growth factor subunit A (PDGF-A), interleukin 6 (IL-6), interleukin 8 (IL-8), and insulin-like growth factor binding protein 2 (IGFBP-2) in 34 surgical samples from UT were analyzed with real-time PCR using the SYBR Supermix Kit (Bio-Rad, Hercules, CA). PCR included the following components: 100 nM each primer, diluted cDNA templates, and iQ SYBR Green supermix and running for 40 cycles at 95°C for 20 s and 60°C for 1 min. PCR efficiency was examined by serially diluting the template cDNA, and the melting curve data were collected to check PCR specificity. Each cDNA sample was run as triplicates, and the corresponding no–reverse-transcriptase (RT) mRNA sample was included as a negative control. The β-actin primer was included in every plate to avoid sample variations. The relative mRNA level was presented as unit values of 2^[Ct(β-actin) – Ct(gene of interest)]. The primer sequences for ZIP4, MMP-9, VEGF-A, PDGF-A, IL-6, IL-8, and IGFBP-2 are listed in Supplementary Table S1.

Statistical Analysis

Statistical analysis was performed using the statistical package SPSS (version 16.0; SPSS Inc., Chicago, IL). For the microarray data, if multiple probes were used for a single candidate gene, the reference sequence of each probe was searched in GenBank and the probes detecting exons were chosen for further analysis. Of the 24 zinc transporters, the probe for ZnT4 was missing in the CGCA database and the probe for ZIP11 was missing in the REMBRANDT database. Microarray expression data of genes were further normalized by subtracting the mean and dividing by the standard deviation for the probe set. The differences of the transcription levels of the 14 ZIPs and 10 ZnTs between grade II and grade IV tumors were compared using Student's *t* test. Both REMBRANDT and CGCA samples were dichotomized into low and high gene-expression groups, with cutoffs set at the median value of the ZIPs and ZnTs expression levels. Overall survival times were compared using the Kaplan-Meier method and formally compared using the log-rank test. The strength of the associations was measured using hazard ratio, which is the ratio of the instantaneous death rate comparing surviving patients with high zinc transporter level with patients having low zinc transporter level. The prognostic value of the 14 ZIPs and 10 ZnTs were evaluated using multivariate Cox proportional hazard regression models, adjusting for patients' tumor grade, age, and preoperational KPS score. Associations between ZIP4 and MMP-9, VEGF-A, PDGF-A, IL-8, IL-6, and IGFBP-2 transcription levels were assessed using simple linear regression models. All statistical tests were 2-sided, and $P < .05$ were considered to be statistically significant. When multiple comparisons were involved, Bonferroni correction was used and the significance cutoff was $.05/24 = .002$.

Results

Gene Profiling Identifies Dysregulated Zinc Transporters in Human Glioma

We analyzed 186 tumor samples from patients with glioma in the CGCA cohort. Of these patients with glioma, 98 (52.7%) received a diagnosis at Glioma Center of Beijing Tiantan Hospital of grade II gliomas and 88 (47.3%) received a diagnosis of grade IV gliomas. The expression levels of the 24 zinc transporters were compared between the grade II gliomas and the grade IV gliomas. The results are shown in the heatmap diagram in Fig. 1. Specifically, after Bonferroni correction for multiple comparisons, ZIP3, ZIP4, ZIP8, ZIP14,

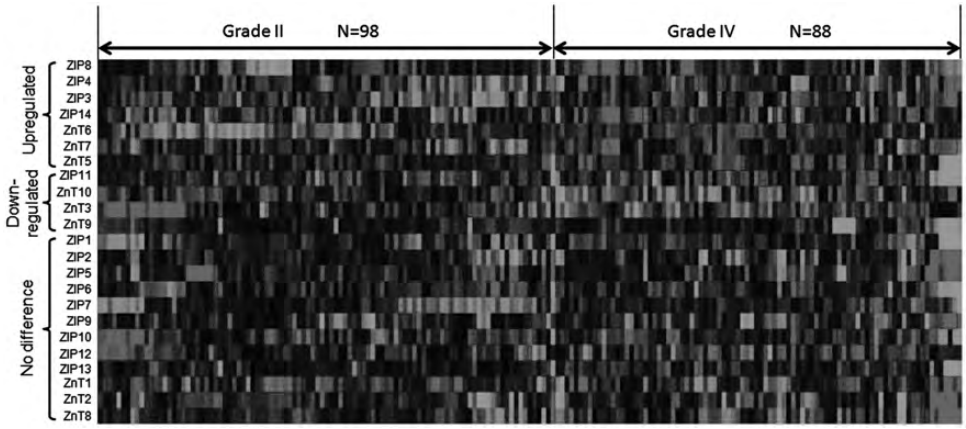

Fig. 1. Heatmap diagram depicting the expression levels of zinc transporters in CGCA cohort. The colors represent the relative levels of gene expression with the brightest red indicating the highest level of expression and green depicting low levels or absence of expression. Data were standardized by subtracting the mean and dividing by the standard deviation for the probe set. Probe sets and samples were arranged and displayed using Cluster and Treeview software. Genes were clustered in 3 groups (upregulated in grade IV, downregulated in grade IV, and no significant difference); in each group, genes were ordered by statistical significance evaluated from Student's *t*-test. If multiple probes were used for a single candidate gene, the reference sequence of each probe was searched in GenBank, and the probes detecting exons were chosen for further analysis. The probe for ZnT4 is missing in the CGCA database, and the probe for ZIP11 is missing in the REMBRANDT database.

ZnT5, ZnT6, and ZnT7 were found to be significantly upregulated in grade IV patients, compared with grade II patients ($P < .002$). On the other hand, ZIP11, ZnT3, ZnT9, and ZnT10 were significantly downregulated in grade IV patients, compared with grade II patients ($P < .002$).

To verify these findings, we also conducted similar analysis for the 335 patients with gliomas in the REMBRANDT cohort, in which 139 (39.2%) and 196 (60.8%) patients received a diagnosis of grade II and grade IV gliomas, respectively. The results on the comparison of the expression levels between the 2 groups are presented in Supplementary Fig. S1. These results are consistent with the findings from the CGCA cohort. Specifically, ZIP1, ZIP3, ZIP4, ZIP7, ZIP8, ZIP13, ZIP14, ZnT5, ZnT6, and ZnT7 were upregulated and ZnT10 was downregulated in grade IV, compared with grade II gliomas ($P < .002$). The consistent findings across the CGCA and REMBRANDT cohorts suggest that specific zinc transporters may play important roles in glioma pathogenesis and progression. A summary of mean differences, 95% confidence intervals (CIs) of differences, and *P* values based on 2 sample *t* test for the CGCA and REMBRANDT cohorts is provided in the Supplementary Table S2.

Association between Zinc Transporters and the Overall Survival Time among Patients with Glioma

To account for the multiple comparison at 24 transporters, Bonferroni correction was used to adjust the significance level, and *P* value <.002 is declared as statistically significant. The hazard ratio of each zinc transporter was estimated from multivariate Cox proportional hazard regression models, and the results are summarized in Table 2. Among the 24 zinc transporters, ZIP4 showed moderate association with patient survival in the CGCA and moderate association in the REMBRANDT cohorts (hazard ratio = 1.61, 95% CI = 1.02–2.53, $P = .040$ based on CGCA cohort and hazard ratio = 1.32, 95% CI = 1.08–1.61, $P = .007$ based on REMBRANDT cohort), and ZIP3 showed significant association in REMBRANDT cohort (hazard ratio = 1.41, 95% CI = 1.16–1.70, $P < .001$), but not significant in CGCA cohort (hazard ratio = 1.22, 95% CI = 0.91–1.63, $P = .181$). ZIP6, ZIP7, ZIP9, ZIP10, ZIP11, ZnT8 and ZnT9 showed significant association with survival in CGCA cohort, but not in REMBRANDT cohort. The minor statistical difference in certain ZIPs (such as ZIP3) between the CGCA and REMBRANDT cohort might be attributable to the different age distribution and ethnicity of the patients from those 2 cohorts.

We further evaluated the prognostic values of ZIP4 by Kaplan-Meier plotting method and compared with log-rank test. ZIP4 showed a significant association with survival both in CGCA cohort (low expression vs high expression, 40.3 months [95% CI, 35.7–45.0 months] vs 29.4 months [95% CI, 25.8–33.0 months]; $P = .009$) and in REMBRANDT cohort (42.5 months [95% CI, 35.3–50.0 months] vs 24.7 months [95% CI, 20.6–28.8 months]; $P < .001$) (Fig. 2). In grade II glioma group, ZIP4 is significantly correlated with patients' survival in REMBRANDT cohort (67.8 months vs 33.2 months; $P < .001$), but not in CGCA cohort because of limited number of deaths. ZIP4 does not correlate with prognosis in grade IV glioma subgroup. Prognostic values of other potential zinc transporters (ZIP3, ZIP6, ZIP7, ZIP9, ZIP10, ZIP11, ZnT8, and ZnT9) were also evaluated (Supplementary Figs. S3–S10).

Validation of the Microarray Data by Real-Time PCR

On the basis of the expression profile and the survival analyses described above, we chose ZIP4 for further

Table 2. Correlation of zinc transporters with patients' survival in CGCA and REMBRANDT database.

	CGCA cohort			REMBRANDT cohort		
Zinc transporter	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
ZIP1	0.813	0.560–1.180	.275	1.115	0.735–1.691	.61
ZIP2	1.545	0.899–2.655	.115	0.685	0.431–1.090	.11
ZIP3	1.22	0.912–1.631	.181	1.407	1.163–1.703	.001
ZIP4	1.606	1.021–2.527	.040	1.318	1.077–1.614	.007
ZIP5	0.87	0.646–1.172	.359	1.039	0.823–1.312	.746
ZIP6	0.651	0.452–0.936	.021	0.83	0.631–1.092	.183
ZIP7	1.343	1.007–1.791	.044	0.952	0.785–1.154	.615
ZIP8	0.851	0.622–1.164	.314	1.039	0.902–1.198	.592
ZIP9	2.93	1.218–7.043	.016	0.73	0.528–1.010	.058
ZIP10	0.506	0.296–0.866	.013	0.93	0.370–2.335	.877
ZIP11	0.702	0.550–0.896	.004			
ZIP12	0.716	0.480–1.068	.101	1.912	0.849–1.207	.891
ZIP13	0.845	0.519–1.378	.500	1.221	1.076–1.387	.734
ZIP14	1.112	0.657–1.883	.692	1.012	0.852–1.202	.893
ZnT1	0.766	0.262–2.235	.625	1.139	0.579–2.242	.706
ZnT2	1.245	0.738–2.100	.412	0.764	0.437–1.333	.343
ZnT3	1.051	0.764–1.447	.759	0.623	0.311–1.249	.183
ZnT4-1				1.042	0.913–1.188	.545
ZnT5	0.761	0.512–1.129	.175	0.965	0.798–1.169	.719
ZnT6	1.008	0.605–1.680	.975	1.082	0.914–1.281	.359
ZnT7	1.205	0.857–1.694	.284	1.04	0.879–1.231	.649
ZnT8	1.532	1.039–2.259	.031	0.946	0.793–1.129	.538
ZnT9	0.769	0.634–0.933	.008	1.135	0.358–3.604	.83
ZnT10	0.874	0.632–1.210	.418	1.042	0.846–1.284	.699

The 24 zinc transporters were correlated with survival time using a separate multivariate Cox regression analysis adjusting for patient's age, preoperational KPS score and tumor grade. The probe for ZnT4 was missing in the CGCA database and ZIP11 was missing in the REMBRANDT database.
Abbreviations: CI, confidence interval; HR, hazard ratio.

validation. ZIP4 mRNA level was examined in 34 surgically resected samples from an independent UT cohort. As shown in Fig. 3, the mean ZIP4 expression level in grade IV glioma is 5.9-fold higher, compared with that of grade II gliomas (Student's *t*-test, $P = .040$). These results, supported by 3 independent cohorts (CGCA, REMBRANDT, and UT), strongly suggest that ZIP4 may be a novel diagnostic and prognostic marker for glioma and may serve as a new target for molecular targeted therapy of glioma.

Correlation between ZIP4 and Other Key Genes for Cell Growth and Angiogenesis in Glioma

To further investigate the underlying mechanism of ZIP4-mediated glioma pathogenesis, we investigated the associations between the expression level of ZIP4 and other key genes for tumor growth. We found that ZIP4 level was significantly associated with several genes that play important roles in cell growth and angiogenesis, such as MMP-9, VEGF-A, PDGF-A, IL-6, IL-8, and IGFBP-2 (Wald test $P < .001$), based on the data from the CGCA cohort (Supplementary Fig. S2). The selection of those 6 genes for validation is based on their expression correlation with ZIP4 levels in the 2 cohorts, and their functional involvement in ZIP4-mediated tumor growth as we previously described.[29,30] To validate this finding, we examined the expression of those 6 genes in 34 surgically resected samples from an independent cohort (UT cohort). As shown in Fig. 4, we plotted the expression level of ZIP4 against each of the 6 genes based on the brain tumor samples from the UT cohort with real-time PCR. The associations were statistically significant (Wald test $P < .001$), suggesting that those genes might play important roles in ZIP4-mediated tumor growth and metastasis of glioma.

Discussion

In this study, we examined the gene profile of zinc transporters (14 ZIPs and 10 ZnTs) in a large sample size of patients with glioma. The associations between the zinc transporters and tumor characteristics (tumor grade and patient overall survival time) were studied by analyzing gene expression data from 3 independent cohorts. We found most of the ZIPs were upregulated in grade IV patients, compared with grade II patients, and ZnTs

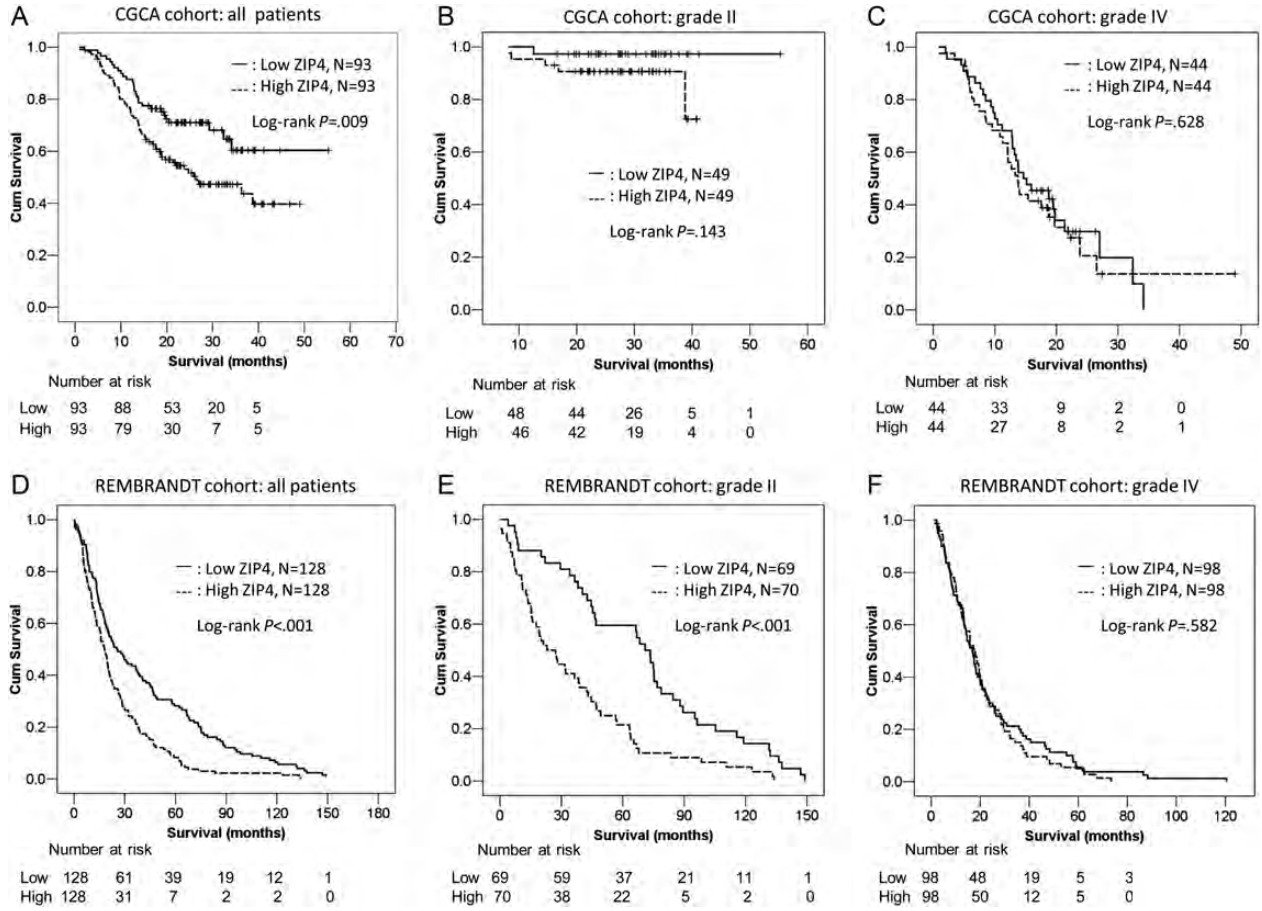

Fig. 2. Kaplan-Meier analyses of overall survival in CGCA and REMBRANDT cohorts according to gene expression level. Discriminative power of ZIP4 in CGCA cohort (A–C) and REMBRANDT cohort (D–F) were assessed with Kaplan-Meier plotting method and log-rank test.

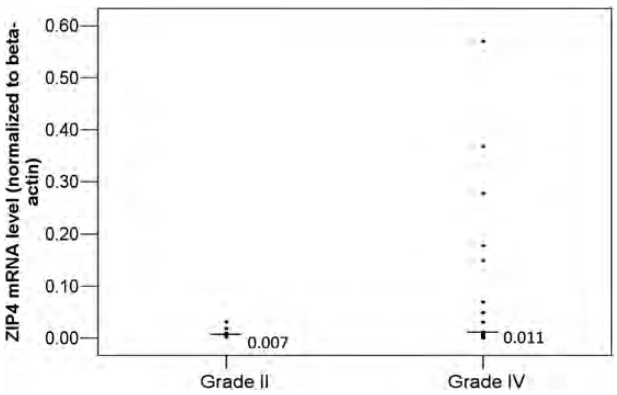

Fig. 3. Quantitative evaluation of ZIP4 expression in UT cohort. Grouped dot plot was used to show the distribution of ZIP4 expression between grade II and grade IV gliomas. The median values for each group is superimposed on these dot plots. There were statistically significant differences between ZIP4 expression levels of grade II gliomas, compared with grade IV gliomas ($P = .040$, Student's t-test).

showed diverse patterns of expression. In survival analysis, cross-validation from CGCA and REMBRANDT cohorts identified ZIP4 as the most significant zinc transporter in glioma pathogenesis and disease progression. High ZIP4 expression was significantly associated with higher grade of gliomas and shorter overall survival time. ZIP4 level was also found to be strongly correlated with key genes for cell growth and angiogenesis in glioma, such as MMP-9, VEGF-A, PDGF-A, IL-6, IL-8, and IGFBP-2.

Upregulation of zinc importers has been identified in many cancer types. Although the exact function of zinc and zinc transporters seem to be cancer-type specific, abnormal zinc level and zinc transporter expressions have been reported to correlate with the progression of cancers. In breast cancer, ZIP6, ZIP7, and ZIP13 are associated with tumor growth and metastasis;[31] in pancreatic cancer, ZIP4 overexpression was linked to enhanced tumorigenesis and progression.[20] In the current study, when looking across CGCA and REMBRANDT cohorts, we found that most of the zinc transporters with altered expression patterns, including ZIP3, ZIP4, ZIP8, ZIP14, ZnT5, ZnT6, and ZnT7, showed positive associations with tumor grade, and only ZnT10 was negatively associated with tumor grade. ZIP3, ZIP4, ZIP8, and ZIP14 are all located on the cellular membrane and facilitate cellular zinc uptake.[17] ZnT5, ZnT6, and ZnT7 are located to the early secretory pathway and may function in transporting cytoplasmic zinc into endoplasmic

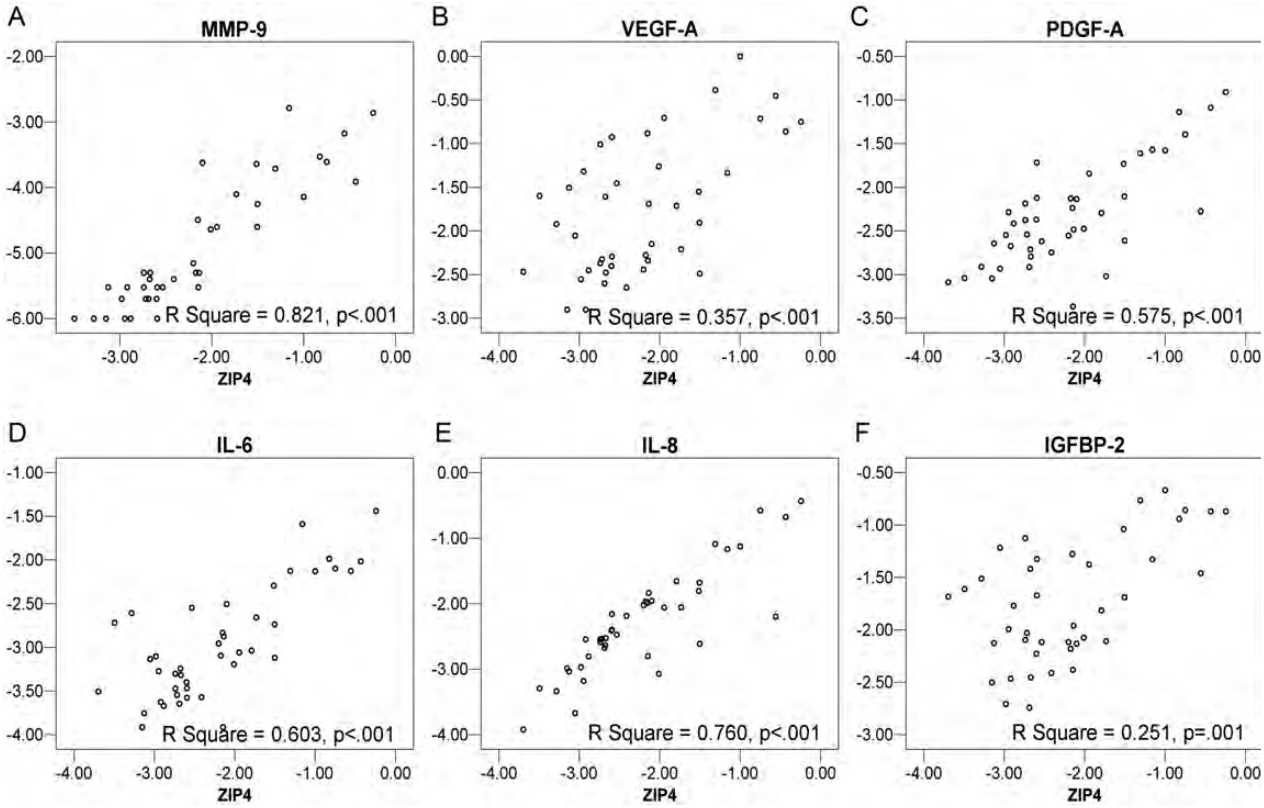

Fig. 4. Correlations between ZIP4 with MMP-9 (A), VEGF-A (B), PDGF-A (C), IL-6 (D), IL-8 (E), and IGFBP-2 (F) levels in UT cohort. The transcription levels of these genes were assessed with real-time PCR and were correlated using linear regression model after log transformation.

reticulum and Golgi apparatus.[17] This finding indicates that gliomas may have enhanced zinc metabolism, and this elevated cellular zinc turnover may be attributed to the tumor malignancy. Among the 24 zinc transporters, only ZnT10 is downregulated in higher grade tumors. ZnT10 is a protein expressed in fetal liver and brain with unclear detailed function, and the pathophysiologic relevance of these alterations remains to be investigated. A limitation of this study is the relatively small sample sizes in the study cohorts; however, we still found several genes with $P < .05$ in the 2 independent cohorts.

ZIP4 is encoded by the *SLC39A4* gene located on chromosome 8q24.3. It was discovered through screening for candidate gene targets responsible for a rare, autosomal recessively inherited disease of intestinal zinc malabsorption, acrodermatitis enteropathica. Patients who receive a diagnosis of this genetic disease present with growth retardation, immune system dysfunction, alopecia, severe dermatitis, diarrhea, and mental disorders.[32] These patients also harbor brain abnormalities that can be ameliorated by zinc supplementation.[33] Our previous studies have shown that elevated expression of ZIP4 protein exists in pancreatic cancer.[20] ZIP4 may promote cancer proliferation and metastasis through regulating the activity of zinc finger transcription factors.[30,34] ZIP4 is also one of the most studied zinc transporters in maintaining zinc homeostasis in humans. Abundant expression of ZIP4 was identified in tissues involved in zinc absorption/reabsorption, such as the gastrointestinal tract and kidney.[17] The exact detailed mechanism of ZIP4 expression regulation is not fully understood. We found that ZIP4 level in glioma is correlated with tumor grade and patient survival. This suggests that ZIP4 overexpression may be involved in the malignant transformation process of this tumor, and ZIP4 may serve as a novel diagnostic and prognostic marker for gliomas.

To expand our knowledge on gliomagenesis, we also examined the expression of ZIP4 in canine glioma tissues. The incidence of canine gliomas is 5 times higher than that in human; canine gliomas share many microscopic, immunoreactive, and molecular features found in human gliomas.[35–38] Of interest, we found that aberrant expression of ZIP4 protein also exists in canine glioma specimens (data not shown). This suggests that ZIP4-driven cancer pathogenesis and progression is a common event not only in different cancer types, but also in different species. It also suggests that canine glioma may be a useful model in studying the role of ZIP4 as a potential therapeutic target.

To further understand the biological implications of ZIP4, we correlated ZIP4 expression with the other 41 090 probes on the microarray of the CGCA database. We found that genes significantly correlated with ZIP4 level are mostly transcription factors, zinc finger proteins, and DNA and RNA binding proteins. There are also genes correlating with ZIP4 level that are highly involved in cell invasion and angiogenesis. Those genes play important roles in tumor aggressiveness and grade of malignancy

and are inversely correlated with patient survival. MMPs are zinc-based proteinases involved in the invasion process.[39] VEGF is one of the most studied molecules involved in angiogenesis and vessel permeability.[40] PDGF is implicated in gliomagenesis and progression,[41] and IL-6, IL-8 and IGFBP-2 are cytokines that have been shown to be related with tumor angiogenesis, progression, and poor prognosis in gliomas.[42–44] The selection of those genes for further validation is based on their expression correlation with ZIP4 levels in these cohorts and their functional involvement in ZIP4-mediated tumor growth, as we previously described.[29,30] We found that ZIP4 expression was significantly correlated with all of these genes in both the CGCA and the UT cohorts, suggesting that ZIP4 may be involved in multiple signaling pathways in gliomagenesis and potentially regulate glioma progression through interacting with the cell growth and angiogenesis via those genes.

In summary, gene profiling of zinc transporters (14 ZIPs and 10 ZnTs) showed that dysregulated zinc and zinc transporters exist in human gliomas. In particular, ZIP4 expression is significantly associated with tumor grade and clinical outcome of patients with glioma. Its overexpression predicts poor clinical outcome and may serve as a promising biomarker for glioma. Very few studies have investigated the functional relevance of zinc transporters in glioma, and it is still not clear how zinc and zinc transporters affect glioma tumorigenesis and aggressiveness. Further studies are warranted on ZIP4 and zinc-regulated biological functions that may provide new insights on glioma pathogenesis and development of new targeted therapies for gliomas.

Supplementary Material

Supplementary material is available online at *Neuro-Oncology* (http://neuro-oncology.oxfordjournals.org/).

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