Polymorphism -433 C>T of the Osteopontin Gene is Associated with the Susceptibility to Develop Gliomas and their Prognosis in a Chinese Cohort

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Key Words
Gliomas • Osteopontin • Polymorphism • Risk • Prognosis

Abstract
Aim: To investigate role of the Osteopontin (OPN) genetic polymorphisms in the susceptibility to gliomas and their prognosis. Methods: A total of 248 Chinese glioma patients and 281 age and sex matched healthy controls were recruited. The genetic polymorphisms at three loci, namely, −156 GG>G, −443 C>T and -66T>G, were determined. The log-rank test and Kaplan–Meier analysis were introduced to assess the effect of OPN gene polymorphisms on patient survival. Results: We found that the genotype frequencies of OPN −443 C>T polymorphism were significantly different between glioma patients and controls. Multivariable analyses showed a higher risk for gliomas in −443 CC genotype carriers compared to -443TT carriers (P<0.001). In addition, we also found the OPN −443 C>T polymorphism was closely related to the gliomas’ tumor grade. The −443 C>T polymorphism also affected the tumor OPN expression level, but not the serum OPN level. More importantly, the −443 C>T polymorphism was significantly associated with the prognosis of these patients regardless of their treatment status. The patients with -443CC genotype had a poorer prognosis than those with -443TT and -443CT genotypes. In contrast, the −156 G>GG and -66T>G polymorphisms were not associated with risk, clinical characteristics, or prognosis of gliomas. Conclusion: This study suggests that the -443C>T gene polymorphisms may be used as a molecular marker for glioma occurrence and clinical outcome in glioma patients.

Z. Shen and B. Chen contributed equally to this manuscript.
Introduction

Gliomas are the most common primary tumors of the central nervous system. Around 70% of gliomas are malignant with high recurrence and mortality rates [1]. The etiology of gliomas remains poorly understood. Some studies suggest positive correlations between susceptibility to gliomas and occupational and environmental exposures [2, 3]. Recent studies revealed that genetic background also contributes to the susceptibility to gliomas [4-6].

Despite advances in diagnostic and therapeutic strategies, the prognosis of gliomas remains poor. A series of factors have been established to affect the prognosis of gliomas, including onset age, Karnofsky performance status (KPS) score, histological grade, tumor necrosis, extent of surgical resection, use of postoperative radiation therapy and adjuvant chemotherapy [7]. The genetic factor also plays a role in the prognosis of glioma patients [8].

Osteopontin (OPN) is a sibling glycoprotein involved in tumor progression and metastasis by regulating cell survival, migration and adhesion [9]. OPN is also implicated in tumorgenesis and has been proposed as a marker for a series of cancers, including lung cancer, ovarian cancer, prostate cancer, and cervical cancer [10-12]. In brain tumors, inhibition of OPN reduces the clonogenic survival in glioma cell lines. Increased plasma OPN has been reported in malignant glioma patients and is associated with poor survival [13]. OPN is associated with neutrophil and macrophage infiltration in glioblastoma [14]. OPN also regulates human glioma cell invasiveness and tumor growth in mice [15] and its expression correlates with angiogenesis and survival in malignant astrocytoma [16].

Several single nucleotide polymorphisms (SNP) in the OPN encoding gene regulate OPN expression. Three loci of OPN gene polymorphisms, including −156 GG>G, −443 C>T and -66T>G, have been identified [17]. OPN genetic polymorphism has been reported to be closely associated with the risk and clinical features of papillary thyroid cancer and cervical cancer in Chinese cohorts [18-20].

To date there is no study addressing the association between OPN genetic polymorphism and the susceptibility to gliomas and their prognosis. Based on the association between OPN protein and gliomas, we postulate that OPN gene polymorphisms may also be related to susceptibility and prognosis of gliomas. In the present study, we enrolled patients with gliomas to test this hypothesis.

Materials and Methods

Patients

This study included 248 glioma patients who underwent surgical resection (gross total tumor resection or partial tumor resection) and adjuvant therapy (radiotherapy, chemotherapy or both) from March 2005 to August 2011. The tumor specimens were obtained during operation and were classified according to the current WHO system (grades: WHO I, WHO II, WHO III and WHO IV) [21]. A total of 281 age and sex matched healthy tumor-free volunteers were recruited from annual checkup visitors as control subjects. All participants were genetically unrelated ethnic Han Chinese people. Each eligible subject was interviewed by two physicians blind to the study protocol to obtain information on demographic factors, family history of cancer, smoking status, and other health characteristics. Follow-up information for all patients was obtained every 3 months by telephone, at a visit or via a posted questionnaire. During the follow-up period, overall survival was measured from diagnosis to death or the last follow-up (5 years). Patients, who died of diseases not directly related to their gliomas or due to unexpected events, were excluded from this study. This study was approved by the institutional review boards of our hospital. All patients gave written informed consent to participate in the present study.

OPN gene polymorphisms

Whole blood was obtained from patients and controls under fasting conditions on the first day of enrollment. DNA was extracted from peripheral whole blood using a Qiagen DNA Isolation Kit (Qiagen,
Valencia, CA, USA). The single nucleotide polymorphisms on the promoter region of OPN gene were determined using TaqMan 5’ allelic discrimination assay. It was performed using the commercially available kit Assays-on-Demand™ from SNP genotyping products (Applied Biosystems, Foster City, CA). SNP amplification assays were used according to the manufacturer’s instructions. In short, 10 ng of sample DNA in 25 µL of reaction solution containing 12.5 µL of the 2× TaqMan® Universal PCR Mix (Applied Biosystems), and 1.25 µl of pre-developed assay reagent from the SNP genotyping product containing two primers and two MCB-Taqman probes. Reaction condition consisted of pre incubation at 50 °C for 2 min, at 95 °C for 10 min, and followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Amplifications were performed in an ABI Prism® 7500 Sequence Detection System (Applied Biosystems) [22].

Western blot assay

The tumor samples were collected from each patient during surgical treatment. Samples were homogenized and lysed. Proteins were resolved by electrophoresis on 8–12% sodium dodecyl sulfate–polyacrylamide gels and transferred by electrobloctting to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat dry milk and incubated overnight at 4 °C with the anti-OPN (Santa Cruz,1:1000), anti-vascular endothelial growth factor A (anti-VEGF-A) (Santa Cruz,1:1000), anti-calpain small subunit 1 (Capn4) (Biocompare,1:1000), anti-NF-kB (Santa Cruz,1:1000), anti-interleukin 1 (IL1) (Santa Cruz,1:1000) and anti-GAPDH (Santa Cruz, 1:2000), antibodies. The anti-human IgG was used as negative control. Immunolabeling was detected using the enhanced chemiluminescence Reagent (Amersham Biosciences).

Serum OPN level detection

The levels of the full length and the thrombin-cleaved OPN in serum were determined by capture enzyme-linked immunosorbent assay (ELISA) according to the protocol provided by the manufacturer (Calbiochem, San Diego, CA). The sensitivity for OPN was 3.33 ng/ml with an intra- and inter-assay coefficients of variation (CV) of <5% and <10%, respectively [23].

Statistical Analysis

The Fisher’s exact Chi-square test was used to compare the frequency distribution of age, gender, smoking status, and body mass index (BMI) between cases and controls, if appropriate. The overall survival (OS) rate was defined as the percentage of patients who were alive 5 years after their diagnosis or the start of treatment. The OS rate was compared by using log-rank test. We performed univariate and multivariate Cox proportional hazard regression analysis to estimate the effect of OPN polymorphisms on survival in the presence of other known prognostic factors. We calculated hazard ratios (HR) and their corresponding 95% confidence intervals. To evaluate the amount of expression, the Raytest TINA software (http://www.raytest.de/service/raytest_catalog.html) was used for densometric analysis of Western blots. Analyses were performed using the software SPSS 16.0 (SPSS Inc., Chicago, IL, USA). All P values were two-sided, and a P value < 0.05 was considered significant.

Results

The characteristics of patients and control

The characteristics of glioma patients and controls are summarized in Table 1. There was no significant difference in the mean age at diagnosis, nor in sex or BMI between glioma patients and controls. However, the patients group had higher percentages for smokers and tumor family history (P=0.021 and P<0.001, respectively).

Association between OPN genotype and risk of gliomas

The genotype frequencies of all studied OPN polymorphisms were in the Hardy-Weinberg equilibrium (all P>0.05). Table 2 shows the genotype and allele frequencies of OPN -443C>T polymorphism was significantly different between the glioma and control groups. The glioma patients had a markedly higher frequency of -443CC genotype than controls (32.66% vs.19.22%, P<0.001). With -443TT as a reference, multivariate logistic regression analysis showed the -443CC genotype carriers had a higher susceptibility to
gliomas (adjusted OR=2.50, adjusted P<0.001) with adjustment for age, sex, smoking status, histology and cancer stage. The C allele carriage represented a higher risk for glioma incidence after adjustment with the above mentioned clinical variables compared with T allele (adjusted OR=1.61, P=0.013). In contrast, the genotypes and allele frequency of -156/G>GG and -66T>G were not significantly different between good responders and poor responders (both P>0.05).

**Glioma histological grades stratified by the OPN genotypes**
We further analyzed the clinical characteristics of gliomas based on the OPN genotypes. We found that the OPN -443C>T was significantly associated with the histological tumor...
grade and KPS score. There was a markedly higher prevalence of -443CC carriers in the high grade subgroup (WHO III+IV) than in the low grade subgroup (WHO I+II) (29.03% vs. 12.90%). Similarly, the -443CC carriers also had lower KPS scores (24.68% vs. 11.76%). Multivariable analyses showed -443CC is correlated to a higher risk for III+IV tumor grade (odds ratio=3.767, P=0.008) and lower KPS scores (odds ratio=2.054, P=0.012) after adjustment with age, sex, BMI, smoking status and family history, using 1772CC as the reference (Table 3). The -156 G>GG and -66 T>G polymorphisms did not affect the clinical characteristics of gliomas (all P>0.05, data not shown).

**OPN gene polymorphisms and serum OPN level**

The mean serum OPN levels were compared according to the OPN genotypes. We found none of the studied polymorphisms affected the serum OPN levels in this study (data not shown). Also, there was no correlation between OPN levels and the clinical features of gliomas, including tumor grade and KPS scores (data not shown).

**Western blot for OPN from tumor samples**

The expression level of OPN and other inflammatory factors were detected by Western blot assay. The quantitative analysis was performed to compare the protein levels in tumors with different genotypes. Figure 1 shows that -443C>T significantly affected the tumor OPN expression levels. The -443CC carriers had a higher OPN expression level than -443CT and -443TT carriers. Meanwhile, we also found that the -443CC genotype carriers had significantly higher VEGF-A, Capn4, NF-κB and IL-1 protein levels than those with -443TC and -443TT genotypes. In contrast, the -156 G>GG and -66 T>G polymorphisms did not affect the expression of above-mentioned factors.

**Follow-up analyses**

Of the 248 patients, 167 patients had complete follow-up data. The log-rank test and Kaplan–Meier analysis were introduced to assess the effect of OPN polymorphism on the patient survival. Figure 2 shows the Kaplan–Meier survival analysis of glioma patients, stratified by the OPN polymorphisms. The -443C>T polymorphism was the only one that affected the prognosis of gliomas. Overall, the -443CC carriers had significantly lower survival rate (33.4%) than those with -443CT (62.5%) and -443CC (63.1%) (Fig. 2a). When all the patients were sub-grouped by treatment status, namely, surgery + chemotherapy, surgery + radiotherapy and surgery+ combination of chemotherapy and radiotherapy, the -443CC was always associated with significantly lower survival rates (all P<0.001, Fig. 2 b,c, and d).

### Table 3. The histological grade according to the genotypes of two loci of HIF-1α gene

| Genotype | III+IV | I+II | adjusted OR | 95% CI | adjusted P |
|----------|--------|------|-------------|--------|------------|
|          | n      | %    | n           | %      |            |
| -443C/T  | TT     | 43   | 23.12%      | 24     | 30.71%     | 1.000      |
|          | CT     | 89   | 47.85%      | 30     | 46.39%     | 1.656 0.866 3.167 0.126 |
|          | CC     | 54   | 29.03%      | 8      | 12.90%     | 3.767 1.540 9.219 0.003 |
|          | T      | 175  | 47.04%      | 78     | 62.90%     | 1.000      |
|          | C      | 197  | 52.96%      | 46     | 37.10%     | 1.909 1.257 2.898 0.002 |
|          | KPS<70 | N=154|             |        |            |            |
|          | KPS>70 | N=94 |             |        |            |            |
| -443C/T  | TT     | 37   | 24.03%      | 25     | 26.60%     | 1.000      |
|          | CT     | 79   | 51.30%      | 59     | 62.77%     | 0.905 0.492 1.664 0.747 |
|          | CC     | 38   | 24.60%      | 10     | 10.64%     | 2.568 1.005 6.079 0.030 |
|          | T      | 153  | 49.68%      | 109    | 57.98%     | 1.000      |
|          | C      | 155  | 50.32%      | 79     | 42.02%     | 1.398 0.970 2.015 0.079 |
Univariate and multivariate Cox proportional hazards regression models were performed to estimate the crude hazard ratios (HRs), adjusted HRs for OS rate in cases and their 95% CIs, with adjustment for age, sex, BMI, family history of cancer, smoking status, histology, WHO grade and therapy status. Compared with the TT carriers of -443C>T, the -443CC genotype carriage represented a poorer prognosis (HR=3.23, 95% CI: 2.49-4.65, P=0.0012).

**Discussion**

In the present study, we investigated the association between OPN gene polymorphism and susceptibility, clinical features and prognosis of gliomas in Chinese patients. We found that the glioma patients had significantly higher rates of -443CC genotypes than controls. The multivariate logistic regression analysis showed a significantly increased risk for gliomas for the -443CC genotype. Also, we found that the high tumor OPN expression was significantly...
more prevalent in -443CC carriers than TT carriers. The -443C>T polymorphism was closely associated with the tumor grade and KPS scores of glioma patients. More importantly, the -443C>T polymorphism was significantly associated with the prognosis of these patients regardless of their treatment status. Our study implies that the OPN -443C>T may be used as a molecular marker for gliomas.

Previous researches have shown that OPN is up-regulated in a variety of cancers. High OPN levels in plasma and tissue were associated with shortened survival of patients with advanced cervical cancer [24]. Low OPN levels were significantly associated with a favorable prognosis with advanced non-small cell lung cancer [25], colorectal cancer [26], upper urinary tract urothelial carcinoma [27, 28], oral squamous cell carcinoma [29], and endometrial cancer [30].

The OPN encoding gene is mapped on human chromosome 4q21-q25 and polymorphisms in the OPN gene promoter can affect its transcriptional activity [31, 32]. More than fifty single nucleotide polymorphisms have been identified in the human OPN encoding gene in different populations, of which three single nucleotide polymorphisms on the promoter region of OPN gene, namely, -66T>G (rs28357094), -156G>GG (rs17524488), and -443C>T (rs11730582), have been extensively studied [31]. A previous study explored the possible role of −443T>C in OPN expression in melanoma cells. The authors found that the -443CC genotype had higher levels of OPN mRNA compared with other allelic variants and -443C>T variants might influence the OPN mRNA levels via binding of c-Myb transcription factor [33]. In oral squamous cell carcinoma (OSCC) patients, more prevalent -443 T/T genotype was found in OSCC patients [34, 35]. Our data showed that -443C>T was
significantly related to the gliomas risk. Additionally, we observed that the -443CC carriers tended to have higher OPN expression in tumors than -443CT carriers or -443TT carriers. This is consistent with the result of a previous study, which explored the promoter activity of the -443 C>T polymorphism using a dual luciferase reporter assay, the authors of which found that a significantly higher luciferase activity was observed in the pGL3-C construct compared to the pGL3-T construct [36]. We also observed that the -443C>T also significantly correlated with tumor grade and KPS scores in this study.

Hou et al reported that the OPN -66T>G, -156G>GG, and -443C>T do not affect the serum OPN level in Chinese patients with hypertension [17]. In our study, we did not find the positive association between these gene polymorphisms with serum OPN levels. However, in the glioma tumor samples, the -443C>T was significantly related to the OPN levels. The -443CC carriers had higher OPN expression levels. In addition, this gene polymorphism was also associated with several factors related to the gliomas invasion and metastasis, including VEGF-A, Capn4, NF-kB and IL, suggesting that the -443 C>T gene polymorphism may affect prognosis via regulation of the inflammatory and pro-angiogenesis factors in gliomas.

One limitation in the present study was that only Chinese patients were enrolled. The associations of OPN gene polymorphism with gliomas needs to be replicated in other ethnic populations. Secondly, the detailed mechanism under which the OPN gene polymorphism affects gliomas remains obscure. Further studies will be required to elucidate these specific mechanisms.

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In the article by Shen et al., entitled “Polymorphism -433 C>T of the Osteopontin Gene is Associated with the Susceptibility to Develop Gliomas and their Prognosis in a Chinese Cohort” [Cell Physiol Biochem 2014;34:1190-1198 (DOI: 10.1159/000366331)], is a printing error in the Table 1.

The corrected Table 1 is stated correctly here.

**Table 1.** The characteristics of case patients and control subjects

| Variables                        | Gliomas (n=248) | Control (n=281) | P     |
|----------------------------------|-----------------|-----------------|-------|
| Sex (male,%)                     | 112 (45.2%)     | 128 (45.6%)     | 0.492 |
| Age at diagnosis (ys)            | 45.4±3.7        | 44.3±4.3        | 0.422 |
| Smoker (%)                       | 80(32.2%)       | 68(24.2%)       | 0.025 |
| BMI (kg/m2)                      | 23.3±2.2        | 22.2±2.5        | 0.104 |
| Family history of cancer         | 46(18.5%)       | 24(8.5%)        | 0.001 |
| Histology                        |                 |                 |       |
| Astrocytomas                     |                 |                 |       |
| Glioblastoma                     | 98              | 150             |       |
| WHO grade                        |                 |                 |       |
| I+II                             |                 |                 |       |
| III+IV                           | 62              | 186             |       |
| Treatment                        |                 |                 |       |
| Surgery only                     |                 |                 |       |
| Surgery + chemotherapy           | 70              |                 |       |
| Surgery + radiotherapy           | 67              |                 |       |
| Surgery + radiotherapy + chemotherapy | 101           |                 |       |
| KPS                              |                 |                 |       |
| <70                              | 154             |                 |       |
| >=70                             | 94              |                 |       |