OCT4 is epigenetically regulated by DNA hypomethylation of promoter and exon in primary gliomas

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Abstract. Glioma is the leading cause of tumor-related mortality in the central nervous system. There is increasing evidence that the self-renewal capacity of cancer cells is critical for the initiation, growth and recurrence of tumors. OCT4 is a transcription factor that plays a key role in regulating the self-renewal ability of embryonic stem cells. DNA methylation is involved in the regulation of OCT4 expression during the development and differentiation of embryonic stem cells and neural stem cells. In the present study, we reported that OCT4 was highly expressed in primary gliomas and its expression levels increased in parallel with pathological grades. BSP analysis showed that the methylation levels of OCT4 gene promoter and exon were significantly reduced in comparison with the normal group and were negatively correlated with OCT4 gene expression in primary gliomas. In vitro, OCT4 gene expression was upregulated following treatment by a demethylation reagent in glioma cell lines. Our findings suggest that OCT4 is epigenetically regulated by DNA hypomethylation in primary gliomas, which may provide evidence for the role of DNA methylation in tumor and may present a new direction for developing more powerful strategies to treat glioma in the clinic.

Introduction

Primary malignant gliomas are diagnosed in 6-7 out of 100,000 people each year. Despite advances in treatment strategies that combine surgery with radiotherapy and chemotherapy, these tumors remain one of the most fatal diseases (1). The genesis mechanisms of gliomas remain unknown.

It was hypothesized in previous studies that the development and prevalent resistance of brain tumor is attributed to brain tumor stem cells (BTSCs), which are a small population of tumor cells within the heterogeneous tumor mass with stem-like characteristics (2). According to this hypothesis, BTSCs sustain the long-term growth of brain tumor and are responsible for tumor recurrence following conventional treatments. Current treatments combining surgery and chemoradiotherapy are not able to eliminate BTSCs (3-5); therefore, new treatments are required to more efficiently target BTSCs. It is necessary to thoroughly understand the molecular and cellular mechanisms underlying the self-renewal and differentiation of BTSCs.

OCT4, also known as OCT3 or POU5F1, is a member of the POU (Pit, Oct, Unc) transcription factor family encoded by the POU5F1 gene (6p2113) and was first identified in 1990 (6). The expression of OCT4 is restricted to pluripotent stem cells and is downregulated when differentiation is initiated during embryonic development. OCT4 is regarded as a gatekeeper at early mammalian development (7) and regulates the self-renewal and pluripotency of human embryonic stem cells (ESCs) (8,9). OCT4 plays an important role in maintaining cellular plasticity and promoting the self-renewal and proliferation ability of stem cells (7). In addition, OCT4-positive cells identified in cancer may represent cancer stem cells (CSCs) and account for the maintenance and propagation of tumors (10,11). In primary glioma, OCT4 is highly expressed and increases in parallel with glioma grading, indicating that the reactivation of OCT4 or other stem cell genes may help to maintain the status of BTSCs and enhance the malignancy of high-grade gliomas (12-14). These findings provide evidence of OCT4 for the stem cell theory on tumorigenesis. However, no study has reported the potential mechanism of how OCT4 expression is upregulated with glioma grading in primary gliomas.

Epigenetic regulation, which refers to the alteration in the expression of heritable genes due to the changes in non-DNA sequences, is one of the crucial mechanisms in the control of gene transcription (15). DNA methylation is a mechanism extensively investigated in this field. The methylation of the CpG island at the promoter usually leads to the downregulation

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of gene expression. Higher level methylation predicts lower transcription activity (16,17). The aberrant DNA methylation pattern can alter gene transcription that is etiologically linked to the formation of cancer (18-20). It was believed that the epigenetic regulation of the chromatin state plays an important role in the control of OCT4 expression (21). DNA methylation of OCT4 gene regulatory regions and histone modifications contribute to the silencing of the OCT4 gene during the differentiation and embryo development of ESCs in mouse and human (22-24). In addition, OCT4 expression to the germ line cells and the early embryo cells may also be mediated by DNA methylation and the mouse ortholog OCT4 is more strongly methylated in differentiated cells than in ESCs (25). OCT4 gene methylation has been studied extensively, but little is known about its DNA methylation at the promoter and exon in primary glioma and its function in tumorigenesis.

Therefore, we hypothesized that DNA methylation may be involved in the regulation of OCT4 expression during the occurrence and development of primary glioma. In this study, we evaluated the expression and the methylation status of OCT4 in 24 patients with primary gliomas and further analyzed their relationship. In vitro, we maintained and treated glioma cell lines with a demethylation reagent to further detect the OCT4 expression.

Materials and methods

Patient selection and sampling. All the investigations described in this study were conducted after informed consent was obtained and in accordance with an institutional review board protocol approved by the Ethics Committee at the Affiliated Hospital of Nantong University. A total of 24 patients with primary glioma were recruited from the Department of Neurosurgery, Affiliated Hospital of Nantong University, from January 2009 to December 2011. Pathological findings were determined by more than 2 pathologists and classified according to the WHO classification standard. There were 12 low- (WHO Grade II) and 12 high-grade tumors (WHO Grade III and IV). The glioma tissues were collected and fixed in 10% formaldehyde followed by embedding in paraffin. In addition, a fraction of samples was placed into liquid nitrogen (-70°C) for use.

Glioma cells and demethylation treatment. Two human glioma cell lines, U87MG and U251MG, were purchased from the Shanghai Cell Institute of the Chinese Academy of Sciences. Both cell lines were maintained in Dulbecco’s Modified Eagle’s Medium; Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin (all from Gibco). Cells were incubated at 37°C in a humidified atmosphere of 5% CO2. Demethylation with 5-Aza-2'-deoxycytidine; when cell cultures reached 50% confluence, they were treated with 5-Aza-2'-deoxycytidine (A3656; Sigma-Aldrich, St. Louis, MO, USA) at the final concentration of 10 nM, respectively, for 3 days.

RNA isolation and real-time PCR. RNA expression levels of OCT4 were determined using quantitative real-time PCR with GAPDH as positive controls. Total mRNA was isolated from glioma specimens and cell lines using mRNA Isolation Kit (Roche, UK) following the manufacturer's instructions. The concentration and purity of mRNA was determined by ultraviolet spectrophotometry. Isolated mRNA (100 ng) from each sample was transcribed to complementary DNA (cDNA) using a First-Strand cDNA Synthesis Kit (Roche), which was then used as a template for quantitative real-time PCR.

Primers used for OCT4 were: 5'-TATTCAGCCAAACGAC CATCT-3' (sense) and 5'-TCAGCTTCTTCCACCCACTT-3' (antisense); for GAPDH, primers were: 5'-GGGAAGCTGT GGCCTGAT-3' (sense) and 5'-AAGGTGGAAGATGGGA GTT-3' (antisense). The primers were designed using Primer 5.0 software and manufactured by TIB MOLBIOL. A 20 µl reaction, which included 2 µl of cDNA template, 2 µl of forward and reverse primer, 6 µl DEPC H2O and 10 µl SYBR-Green Mix (QPK-201, Toyobo), was conducted using the ABI Prism 7500 Sequence Detection System (Applied Biosystems). PCRs of each template were performed in duplicate in a 96-well plate. The thermal cycling conditions included an initial denaturation step at 95°C for 5 min and 40 cycles at 95°C for 10 sec, at 59°C for 15 sec and at 72°C for 20 sec. The relative fold-change 2-ΔΔCt method was used to determine the relative quantitative gene expression compared with GAPDH. The transcription level of target genes observed in calibrating samples was treated as the basal level and given the value 1.0. All PCR reactions were performed in triplicate and a negative control was included that contained primers without cDNA.

Western blot analysis. The samples were then homogenized in lysis buffer (1% NP-40, 50 mmol/l Tris, pH 7.5, 5 mmol/l EDTA, 1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 1 mmol/l PMSF, 10 mg/ml aprotinin and 1 mg/ml leupeptin; Sigma-Aldrich) and clarified by centrifuging for 20 min in a microcentrifuge at 4°C. Following determination of its protein concentration with the Bradford Assay (Bio-Rad, USA), the resulting supernatant (50 µg of protein) was subjected to SDS-polyacrylamide gel electrophoresis (PAGE). The separated proteins were transferred to a polyvinylidene difluoride membrane (Millipore Corp., USA) by a transfer apparatus at 350 mA for 2.5 h. The membranes were first blocked and then incubated with the primary antibody described above for 2 h at room temperature. After washing three times, filters were incubated with horseradish peroxidase-conjugated human anti-mouse or anti-rabbit antibodies (Pierce) for 1 h at room temperature. Immunocomplexes were detected with an enhanced chemiluminescence system (NEN Life Science Products, USA). The western blotting experiments were repeated at least three times.

Immunohistochemistry (IHC). Each glioma was immunohistochemically examined for OCT4 nuclear staining. Specimens were cut serially into 4-µm sections. Sections were dewaxed in xylene and rehydrated in graded ethanol. Endogenous peroxidase activity was blocked by immersion in 0.3% methanolic peroxide for 30 min. Immunoreactivity was enhanced by microwaving and incubating the tissue sections for 10 min in 0.1 mol/l citrate buffer. Primary rabbit polyclonal antibodies OCT4 (1:200; Abcam, USA) were applied and incubated overnight at 4°C. Secondary Goat Anti-Rabbit immunoglobulin (Invitrogen Life Technologies, USA) was applied and incubated
for 1 h at room temperature. Some sections were processed with Tris-buffered saline rather than primary antibody and were used as negative controls. Immunocomplexes were visualized by brown pigmentation via a standard 3,3-diaminobenzidine (DAB) protocol. Appropriate positive and negative controls were used. Ten high-power fields were randomly chosen and at least 300 cells were counted per field. Tumors were scored as a percentage of positive cells. The staining procedures were repeated at least three times.

DNA preparation and bisulfite genomic sequencing. Genomic DNA was extracted from frozen tissues by proteinase K digestion and the phenol-chloroform method (26). Sodium bisulfite treatment of the extracted DNA was performed as previously described with some modifications (27). In brief, 10 µg DNA in 50 µl TE was incubated with 5.5 µl of 0.3 M NaOH at 37˚C for 15 min and at 95˚C for 2 min and then subjected to sodium bisulfite chemical treatment (2.4 M sodium metabisulfite; 0.5 mM hydroquinone, pH 5.0; both from Sigma-Aldrich). Following incubation for 4 h at 55˚C, the treated DNA was purified using the SK1261 kit (Shenggong Biotechnology Co., China), desulfonated in 0.3 M NaOH and then neutralized to pH 7.0 using 3 M sodium acetate (pH 5.2). The neutralized DNA was then purified using the SK1261 purification kit again, dissolved in TE buffer (pH 8.0).

The methylation status of the minimal promoter and part of exon 1 region CpG islands of OCT4 was analyzed (23,28). The primers were designed to amplify the promoter and exon 1 from -234 to +46 for bisulfite genomic sequencing. The forward primer was 5'-GGATTTGTATTGAGGTTTTGGAG-3' and the reverse, 5'-TAACCCATCACCTCCACCAC-3'. Touchdown PCR was then carried out. An initial denaturation at 94˚C for 4 min was followed by five PCR cycles of 94˚C for 45 sec, 68˚C for 45 sec and 72˚C for 1 min. The PCR was then completed with 35 cycles of 45 sec at 95˚C, 45 sec at 58˚C. The amplified products were gel-purified using the SK1261 kit and then subjected to TA-cloning using pUC18-T vector (Shenggong Biotechnology Co.). Ten clones for each case were selected for sequencing using BigDye version 3.1 and analyzed on automated DNA sequence analyzer (ABI Prism 3730; Applied Biosystems, Inc., Foster City, CA, USA). The cytosine or thymine residues at the CpG sites represented methylated or unmethylated status, respectively.

Statistical analysis. Statistical analysis was performed using SPSS 13.0 for Windows. The Student's independent-sample t-test statistics was used for statistical comparison. The correlation between methylation status and mRNA expression level of OCT4 was tested by Pearson's correlation test. All statistical tests were calculated in two-sided and a P-value <0.05 was considered to indicate a statistically significant difference.

Results

OCT4 is highly expressed in primary gliomas. The primary glioma samples were collected from 24 patients and placed in liquid nitrogen, followed by detection of OCT4 mRNA expression by real-time PCR. Results revealed that expression of OCT4 mRNA was upregulated in all 24 human glioma samples, but no obvious expression was observed in the normal brain samples. Densitometric evaluation of relative expression showed that the level of OCT4 mRNA was significantly higher in high-grade gliomas than in low-grade gliomas (P<0.05) (Fig. 1A). Western blot analysis confirmed that the level of OCT4 protein was higher in primary high-grade gliomas than in low-grade ones (Fig. 1B).

OCT4 protein is localized in the nuclei of primary glioma cells. The primary glioma tissues were collected from 24 patients and paraffin-embedded sections were obtained for IHC, followed by counting of OCT4-positive cells. OCT4 was expressed in the nuclei of tumor cells and OCT4-positive cells were identified in all glioma samples. The percentage of OCT4-positive cells ranged from 3.79 to 79.67%. The nuclei of most high-grade glioma cells (Fig. 2C and D) were more intensely stained than those of low-grade tumors (Fig. 2A and B), indicating that OCT4 expression in the nuclei of glioma cells was in a grade-dependent manner.

Hypomethylation of the OCT4 gene and negative correlation with OCT4 expression in primary glioma. Bisulphate sequencing analysis was employed to examine whether the OCT4 expression...
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was correlated with the hypomethylation of promoter and exon. A total of 11 CpG dinucleotides were measured through BSP sequencing. The minimal promoter region and the exon region contained 5 and 6 CpG dinucleotides, respectively (Fig. 3A). DNA methylation of the OCT4 gene was found in all samples. The CpG dinucleotides in the normal group were highly methylated. However, the DNA methylation level was reduced with the upgrading of the glioma malignancy (Fig. 3B). Statistical analysis showed that DNA methylation level in primary gliomas was significantly lower than that in the normal group (P<0.05). The percent of methylation in high-grade gliomas was significantly lower than that in low-grade ones (P<0.05)(Fig. 3C).
Our findings are consistent with previous studies that high OCT4 mRNA and OCT4 protein were highly expressed in primary gliomas and the levels were grade-dependent. Real-time PCR, western blot and IHC analyses showed that OCT4 mRNA and OCT4 protein expression is detectable since its level is consistent with the expression profile (Fig. 1), the DNA methylation level was negatively correlated to the expression of OCT4 mRNA ($r = -0.156$, $P<0.05$).

**Upregulation of OCT4 in glioma cells following demethylation.** The glioma cell lines (U87MG and U251MG) were treated with 5-Aza-dc, a demethylation reagent, for 72 h. The results showed that OCT4 mRNA expression was upregulated 3.38-fold in U87 cells and 2.35-fold in U251 cells (Fig. 4A). The changing tendency of protein expression was similar to that of mRNA expression (Fig. 4B).

**Discussion**

It is considered that most human primary malignant brain tumors contain BTSCs. These cells are the determinants for the occurrence, development and recurrence of gliomas and for the therapeutic response of brain tumor (29,30). BTSCs provide a novel logical explanation for the drug resistance and the high recurrence rate of primary malignant brain tumors. Since BTSCs are similar to neural stem cells in molecular marker expression and multi-lineage differentiation potential, BTSCs can be studied indirectly in the absence of the default marker for screening BTSCs. We hypothesize that the expression level of genes related to stem-like cells (SLCs) can substitute the rate of SLCs in glioma. SLC-related genes usually include Nes (nestin), CD133 (prominin), ABCG2 (ATP-binding cassette superfamily G member 2), SOX2 (SRY-box containing gene 2), OCT4 (POU class 5 homeobox 1) and Msi-1 (musashi-1). In the present study, we selected OCT4 as the representative of SLC-related genes.

During the tumorigenesis of some CSCs in adults, OCT4 expression is detectable since its level is consistent with the number of CSCs and is closely related to the malignant degree, development and prognosis of tumors (31). In this study, real-time PCR, western blot and IHC analyses showed that both OCT4 mRNA and OCT4 protein were highly expressed in primary gliomas and the levels were grade-dependent. Our findings are consistent with previous studies that high-level OCT4 increased the malignancy of ESC-derived tumors (12,14,31,32). Moreover, OCT4 expression is regarded as a ‘stem cell survival’ factor pattern and is restricted to pluripotent cells (7,33). Our results also supported the BTSC theory of glioma, which suggested that SLCs were present in neoplasm and the malignancy of glioma may be related to the abundance of SLCs in the tumor.

Numerous studies indicate the DNA methylation of the OCT4 gene at the gene regulatory region is a key regulatory factor in OCT4 transcription (22-24,34-36). Cantz et al (37) also argued that the most appropriate method to determine OCT4 expression in cells is to analyze the methylation status of the promoter. Therefore, to explore whether the DNA methylation of OCT4 promoter is involved in the changes of OCT4 expression in primary gliomas, BSP sequencing was employed to measure the methylation of the minimal promoter region and exon region (-234 ~ +46). We found that the methylation levels of the OCT4 gene were different between primary glioma and normal brain tissues. The methylation level in tumor was markedly reduced as compared to the normal group and was lower in high-grade gliomas than in low-grade ones. The results showed that methylation status may regulate the transcription of the OCT4 gene and were involved in glioma development. OCT4 promoter demethylation has already been reported in other types of human cancer. It was also suggested that OCT4 promoter demethylation contributes to tumorigenesis (38,39). Since the demethylation of the OCT4 promoter occurred predominantly in high-grade glioma, OCT4 may contribute to the initiation and progression of glioma and potentially serve as a biomarker for the prognosis of human glioma.

Genomic hypomethylation can cause genome instability and proto-oncogene formation, which leads to high expression (40). Our study showed that the expression of the OCT4 gene was significantly upregulated in glioma samples, as compared with the normal brain samples. Notably, the percentage of promoter methylation in the OCT4 gene was inversely correlated with the expression levels of the OCT4 gene in glioma. In vitro, 5-Aza-dc reactivated the expression of OCT4 in glioma cells. These results suggested that aberrant promoter hypomethylation of OCT4 plays a role in the reexpression of its normal silence. Fanelli et al (41) reported that DNA hypomethylation in GBMs is in part explained by a correlating decrease in expression of de novo DNA methyltransferase DNMT3b. This global change in methylation leads to the reactivation and remobilization of repeat elements satellite 2, D4Z4 and Alu elements, resulting in increased copy number alterations of genomic regions in proximity to these elements (42). However, with regard to OCT4, the potential specific mechanism of hypomethylation in primary glioma requires further study.

In summary, OCT4 is epigenetically regulated by methylation in primary glioma. The methylation status of the OCT4 gene can be regulated by a demethylation reagent. Our findings provide evidence for the role of DNA methylation in primary glioma and present a direction for developing more powerful strategies to treat glioma in the clinic.

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