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DNA methylation level of promoter region of activating transcription factor 5 in glioma*

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Abstract: Transcription factors, which represent an important class of proteins that play key roles in controlling cellular proliferation and cell cycle modulation, are attractive targets for cancer therapy. Previous researches have shown that the expression level of activating transcription factor 5 (ATF5) was frequently increased in glioma and its acetylation level was related to glioma. The purposes of this study were to explore the methylation level of ATF5 in clinical glioma tissues and to explore the effect of ATF5 methylation on the expression of ATF5 in glioma. Methylation of the promoter region of ATF5 was assayed by bisulfite-specific polymerase chain reaction (PCR) sequencing analysis in 35 cases of glioma and 5 normal tissues. Quantitative real-time PCR (qRT-PCR) was also performed to detect ATF5 mRNA expression in 35 cases of glioma and 5 normal tissues. Clinical data were collected from the patients and analyzed. The percentages of methylation of the ATF5 gene in the promoter region in healthy control, patients with well-differentiated glioma, and those with poorly differentiated glioma were 87.78%, 73.89%, and 47.70%, respectively. Analysis of the methylation status of the promoter region of the ATF5 gene showed a gradually decreased methylation level in poorly differentiated glioma, well-differentiated glioma, and normal tissues (P<0.05). There was also a significant difference between well-differentiated glioma and poorly differentiated glioma (P<0.05). ATF5 mRNA expression in glioma was significantly higher than that in the normal tissues (P<0.05). This study provides the first evidence that the methylation level of ATF5 decreased, and its mRNA expression was evidently up-regulated in glioma.

Key words: DNA methylation, Activating transcription factor, Promoter region, Epigenetic, Glioma
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1 Introduction

Activating transcription factor 5 (ATF5) is a member of the cyclic adenosine monophosphate (cAMP)-dependent ATF/cAMP response element binding protein (CREB) families involved in diverse physiological functions, including metabolite homeostasis, cellular proliferation, differentiation, apoptosis, and regulation of the cell cycle (Mora *et al.*, 2001; Mason *et al.*, 2005). ATF5 is an anti-apoptotic protein that is highly expressed in diverse cancers, including those cancers of prostate, rectal, colon, endometrium, breast, ovary, pancreas, gastric, lung, and B-cell chronic lymphocytic leukemia, in particular glioblastoma (Greene *et al.*, 2009; Sheng *et al.*, 2010a; Wu *et al.*, 2014), and contributes to a malignant phenotype. Targets of ATF5 include *ALDOB* (Pascual

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et al., 2008), *ID1* (Gho *et al.*, 2008), *EGR-1* (Li *et al.*, 2009; Liu *et al.*, 2011), *MCL-1* (Sheng *et al.*, 2010a), *HSP27* (Wang *et al.*, 2008), *BCL-2* (Dluzen *et al.*, 2011; Chen *et al.*, 2012), and *CYP2B6* (Pascual *et al.*, 2008). However, whether these genes are ATF5 targets mediating ATF5-dependent cell survival and proliferation remains unclear (Greene *et al.*, 2009; Haakenson *et al.*, 2012).

Glioma is a type of central nervous system (CNS) cancer affecting the glial cells. Glioma is the most frequent (about 40%) type of primary brain tumors, with an average of worldwide annual occurrence close to 190 000 cases, resulting in more than 140 000 deaths each year. Despite major efforts to reduce deaths caused by this disease, the mean survival time of newly diagnosed malignant glioma patients remains at approximately 12 months, and after 24 months of surgical resection, nearly 90% of patients are dead (Singh and Paterson, 2000; Doolittle, 2004). ATF5 has been considered as hallmark of malignant glioma, as it is specifically and highly expressed in human malignant glioma and promotes the proliferation of tumor cells (Hu *et al.*, 2012; Wang *et al.*, 2012).

DNA methylation is involved in the regulation of many cellular processes, including gene transcription, chromosome stability, chromatin structure, X chromosome inactivation, and embryonic development. About 1% of the genome consists of 500–2000 bp CpG-rich areas or islands. Methylation of CpG islands involves the course, in which DNA methyltransferases (DNMTs) transfer a methyl group from *S*-adenosyl-L-methionine to the fifth carbon position of cytosine. DNA methylation is an important epigenetic mechanism for gene silencing and cancer progression (Herman and Baylin, 2003). Aberrant DNA methylation is mainly found in 5'-CpG-3' dinucleotides within promoters or in the first exon of genes, which is an important pathway for the repression of gene transcription in diseased cells. Thus, detection of methylation in some genes of diseased cells could provide very useful information for discrimination of that disease. However, there has been no relevant report about the methylation status and the mRNA expression of ATF5 in glioma and normal brain tissues. The purposes of this study were to explore the correlation of the methylation level of ATF5 gene in glioma and its significance. Our findings may provide evidence for further elucidation of change in the epigenetic modification of ATF5 in glioma.

2 Materials and methods

2.1 Clinical samples

All procedures described in the study were reviewed and approved by the Ethical Committee of Qingdao University, Qingdao, China. Glioma (WHO grades I–IV) tissues collected from 35 patients who underwent surgical resection and 5 normal tissues with acute brain trauma were obtained sequentially from the Affiliated Hospital of Qingdao University (Qingdao, China) in 2014. Glioma of pathologic grades I–II was classified as low-grade one (well-differentiated glioma) and that of pathologic grades III–IV as high-grade one (poorly differentiated glioma). Tissue samples were immediately flash-frozen in liquid nitrogen after resection. All patients and donors gave their informed consent.

2.2 Prediction of CpG islands and design of bisulfite-conversion-based methylation polymerase chain reaction (PCR) primers

The prediction of CpG islands and the design of bisulfite-conversion-based methylation PCR primers of the promoter region of the ATF5 gene were carried out with online tools: EMBOSS Cpgplot (http://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot) and MethPrimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi), respectively.

2.3 Bisulfite sequencing

Genomic DNA, isolated from clinical tissues by using the TIANamp Genomic DNA Kit (Tiangen, China), was treated with bisulfite using the Methylamp™ DNA Modification Kit (Epigentek, USA) according to the manufacturer's instructions. PCR was performed using bisulfite-conversion-based methylation PCR primers: forward, 5'-TGATAGTA GGTGTTGGATAGTTTAA-3'; reverse, 5'-AACCA AAATAATCTCAATTTCCTAAC-3'. Separated PCR products using the Gel Extraction Kit (OMEGA, USA) were cloned into TA-cloning vectors (TaKaRa, Dalian, China). Six positive clones of each sample were selected randomly for DNA sequencing at BGI (Beijing Genomics Institute, China).

2.4 Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from tissues using RNA Fast 200 Kit (FASTAgen, Shanghai, China) according to the manufacturer's instructions. First-strand complementary DNA (cDNA) was synthesized by using a PrimeScript™ RT reagent kit with genomic DNA (gDNA) Eraser (TaKaRa, Dalian, China). In accordance with the manufacturer's instructions, the reverse transcription was performed at 37 °C for 15 min, 85 °C for 5 s in a total volume of 20 μl reaction mix for qPCR. qRT-PCR was carried out using a SYBR Premix Ex Taq™ Kit (TaKaRa, Dalian, China) with *β-actin* as the reference gene. The sequences of the primers were as follows: forward, 5'-AAGT CGGCGGCTCTGAGGTA-3' and reverse, 5'-GGA CTCTGCCCGTTCCTTCA-3' for ATF5; forward, 5'-TGGAACGGTGAAGGTGACAG-3' and reverse, 5'-GGCTTTTAGGATGGCAAGGG-3' for *β-actin*. Data were collected and analyzed by Bio-Rad iQ5 (Bio-Rad, Hercules, CA).

2.5 Statistical analysis

Comparison of DNA methylation level of the ATF5 gene of three groups including poorly differentiated glioma, well-differentiated glioma, and normal samples was carried out using analysis of variance (ANOVA) followed by Student-Newman-Keuls. All statistical analysis was performed using SPSS Statistics 17.0 (SPSS Inc., Chicago, USA). $P<0.05$ was considered statistically significant.

3 Results

3.1 Analysis of the methylation levels of ATF5 in glioma and normal tissues using bisulfite-specific PCR (BSP) sequencing

We found one typical CpG island in the promoter region of ATF5 by EMBOSS Cpgplot, and designed a pair of bisulfite-conversion-based methylation PCR primers using MethPrimer for the region from −1352 to −1160 bp (the transcription initiation site of ATF5 was designated as 0), which contains 9 CpG sites (Fig. 1). Meanwhile, genomic DNA was treated with bisulfite, and amplified by PCR, and the PCR products collected from each tissue were cloned into a sequencing vector and subjected to bisulfite sequencing (Fig. 2).

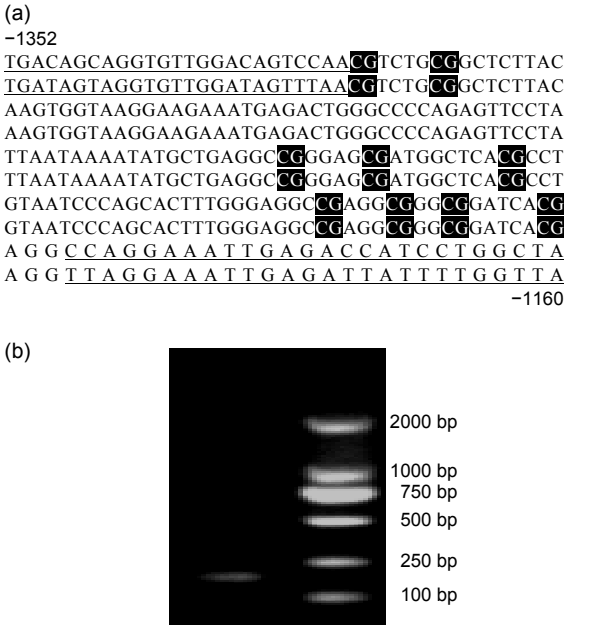

Fig. 1 Nucleotide sequences of promoter region of ATF5 gene (−1352 to −1160 bp) (a) and products from PCR after bisulfite treatment (b)

Two strands in (a) represent the original nucleotide sequences of the promoter region of the ATF5 gene (upper strand) and bisulphate-converted sequences (lower strand), respectively. Primers were underscored and 9 CpG sites in the sequence are marked in black background

Statistical analysis showed that the percentage of methylation of the promoter region of the ATF5 gene was 87.78%, 73.89%, and 47.70% in normal tissues, low-grade glioma, and high-grade glioma, respectively. Analysis of sequencing results revealed that the rate of methylation of the promoter region of ATF5 decreased significantly in high-grade glioma and low-grade glioma compared with normal tissues (Figs. 3a and 3b). Meanwhile, the methylation level of CpG loci of ATF5 was higher in normal tissues, while it was decreased in glioma (Fig. 3c).

3.2 Expression of ATF5 in glioma and normal tissues

To explore the methylation level of ATF5 on its expression in glioma and normal tissues, the transcriptional levels of ATF5 were qualified with qRT-PCR assays in 35 cases of glioma and 5 normal tissues. The average expression levels of ATF5 mRNA were significantly higher in glioma than those in the normal tissues ($P<0.05$; Fig. 3d). These results support the hypothesis that the expression level of ATF5 is associated with its methylation status.

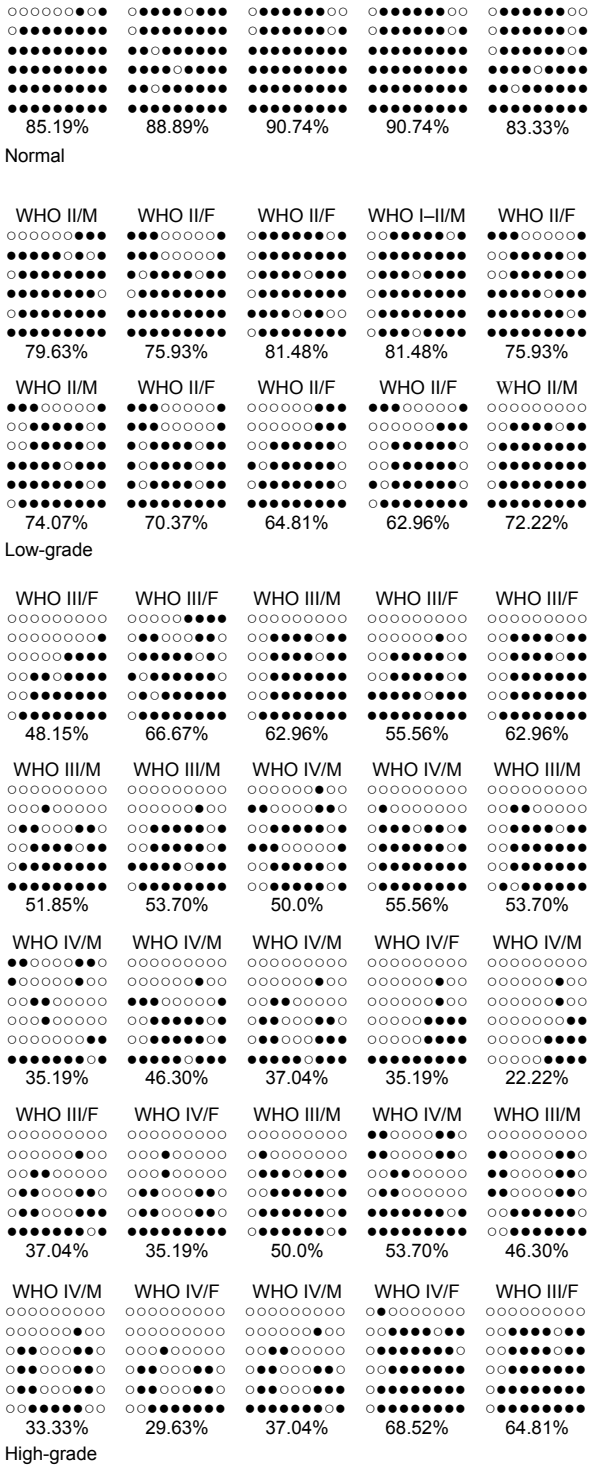

Fig. 2 Bisulfite sequencing results of all tissues, including normal samples, low-grade glioma, and high-grade glioma

Each line represents an individually sequenced clone. White and black circles denote unmethylated and methylated CpG sites, respectively. Percentage of methylation is shown at the bottom

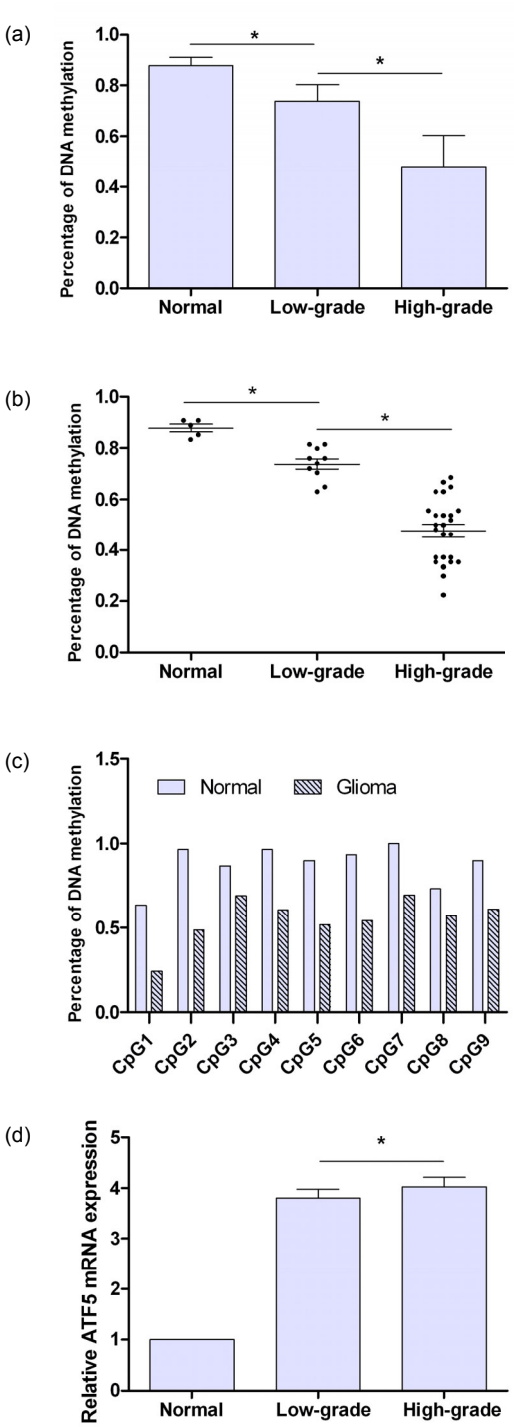

Fig. 3 DNA methylation level of promoter region of the ATF5 gene and mRNA expression of ATF5 in all tested samples

(a) Percentage of methylation level of ATF5 in three groups. (b) Percentage of methylation level of ATF5 in all tested tissue samples. (c) Comparison of methylation levels of 9 CpG sites in the promoter region of ATF5 in glioma and normal tissues. (d) Relative mRNA expression of ATF5 was higher in glioma tissues than in normal tissues. * $P<0.05$

4 Discussion

DNA methylation, which is the most well-researched epigenetic mechanism, plays an important role in gene regulation and the development of life (Razin, 1998). Hypermethylation of tumor suppressor genes and demethylation of oncogene appear to be early molecular events for tumorigenesis and metastasis (Adrien *et al*., 2006). Previous reports from several laboratories demonstrated that an increase of ATF5 expression level is frequently associated with cancers, especially glioma (Sheng *et al.*, 2010b). Our previous results indicated that the acetylation level of ATF5 is related to glioma, but the DNA methylation status of ATF5 and its contributions to the over-expression of ATF5 in glioma are not well-understood. CpG island prediction results indicated that one typical CpG island was found in the ATF5 promoter region. Comparison results of the methylation level of ATF5 indicated that it was relatively highly methylated in normal tissues (87.78%), and decreased significantly in well-differentiated glioma (73.89%) and poorly differentiated glioma (47.70%). Moreover, comparison of the methylation level of 9 CpG sites revealed that the methylation level of the ATF5 promoter region in normal tissues was higher than that in glioma. Our study indicated that a significant difference by Student-Newman-Keuls existed in the methylation level of ATF5 promoter between glioma (poorly differentiated glioma and well-differentiated glioma) and normal tissues. Furthermore, we found that the ATF5 mRNA expression level was significantly higher in glioma than in the normal tissues. It suggests that the methylation status of ATF5 plays an important role in its expression level, so aberrant methylation of ATF5 in glioma may be an important clue in understanding the progression of glioma.

ATF5 is an important anti-apoptotic protein, which is highly expressed in malignant glioma (Kim *et al.*, 1999). Inhibition of the expression of ATF5 can cause apoptosis in rat and human malignant glioma cells, but not in normal brain tissues (Yurochko *et al.*, 1997). ATF5 has a well-established pro-survival activity. Functional experiments demonstrated that the pro-survival function of ATF5 results from its ability to inhibit apoptosis (Dluzen *et al*., 2011). Demethylation of the promoter region of ATF5 may result in its over-expression in glioma, thereby inhibiting apoptosis of cancer cells and then accelerating the progression of glioma. Currently, research mainly focuses on the downstream targets of ATF5 (Wang *et al*., 2014), but the effect of methylation on the ATF5 targets mediating ATF5-dependent cell survival, proliferation, and the possible downstream pathways involved in the development of glioma should be given more attention.

Our findings have shown that the methylation level of ATF5 has significant relevance between normal tissues and glioma ($P<0.05$), and the methylation status of ATF5 plays an important role in its expression. These findings provide evidence for further investigation on the epigenetic modifications in glioma.

Compliance with ethics guidelines

Xiao-min HUA, Juan WANG, Dong-meng QIAN, Jing-yi SONG, Hao CHEN, Xiu-li ZHU, Rui ZHOU, Yu-dan ZHAO, Xiu-zhi ZHOU, Ling LI, Li ZHANG, Xu-xia SONG, and Bin WANG declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study. Additional informed consent was obtained from all patients for whom identifying information is included in this article.

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中文概要

题　目：激活转录因子 5 启动子区甲基化水平的研究

目　的：研究临床胶质瘤标本中激活转录因子 5（ATF5）启动子区 CpG 岛甲基化状态及临床意义。

创新点：首次发现在胶质瘤标本中 ATF5 的甲基化水平下调，其表达水平下调。

方　法：收集 35 临床胶质瘤组织及 5 例急性脑外伤组织作为对照，应用亚硫酸盐测序技术检测 ATF5 的甲基化水平，并结合临床病理资料进行分析；实时荧光定量聚合酶链式反应（qRT-PCR）检测所有标本中 ATF5 mRNA 的表达水平变化。

结　论：5 例正常脑组织、10 例低级别胶质瘤及 25 例高级别胶质瘤的甲基化比例分别为 87.78%、73.89%和 47.70%（图 2），两组相比差异有统计学意义（P<0.05；图 3a 和 3b）；qRT-PCR 结果表明，与对照相比，胶质瘤标本中 ATF5 表达水平上升（P<0.05；图 3d）。综上所述，胶质瘤组织中 ATF5 基因启动子区 CpG 岛的甲基化状态对该基因的表达有重要意义。

关键词：DNA 甲基化；激活转录因子；启动子；表观遗传学；胶质瘤