Promoter hypermethylation is not the major mechanism for inactivation of the *FBXW7* β -form in human gliomas

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FBXW7 has been reported to be a candidate tumor suppressor gene on 4q31. Three isoforms (α -form, β -form, and γ -form) of *FBXW7* are produced from mRNAs with distinct 5' exons. Our previous study identified the specific suppression of the mRNA expression of the *FBXW7* β -form in human gliomas. Because this form is the major *FBXW7* isoform in the human brain, we elucidated the silencing mechanisms for the *FBXW7* β -form in gliomas. No genetic alterations were found in the whole *FBXW7* gene including putative promoter region of the β -form. Treatments with 5-azacytidine and trichostatin A did not induce re-expression. A sodium bisulfite-modification assay indicated that CpG sequences in the promoter of *FBXW7* β -form were not methylated in glioma cells. Meanwhile we searched for the expression of *FBXW7* and the sodium bisulfite sequences in normal human peripheral blood cells, and we surprisingly found that the mRNA expression of the *FBXW7* β -form was highly suppressed and the CpG sequences in the promoter region of the *FBXW7* β -form were heavily methylated. Our data suggest that the inactivation of the *FBXW7* β -form plays an important role in the pathogenesis of gliomas and that an unknown mechanism(s) other than mutation and methylation is the major cause of the suppression of the *FBXW7* β -form in gliomas.

Key words: *FBXW7*, human glioma, methylation, peripheral blood cells

INTRODUCTION

FBXW7 (F-box and WD40 domain protein 7) is a member of the F-box family of proteins, which are phosphopeptide-specific alternative substrate recognition subunits of SCF (Skp1–Cul1–F-box protein) ubiquitin ligases and catalyze the ubiquitination of proteins such as CCNE1, MYC, AURKA, NOTCH1, and JUN, which promote cell proliferation, and are frequently activated in a wide range of human cancers (Nakayama and Nakayama, 2005). Substrate recognition of *FBXW7* is mediated through interaction with seven adjacent WD40 protein binding motifs located in the C-terminus (Orlicky et al., 2003). The role of *FBXW7* as a negative regulator of oncoproteins has suggested that it may function as a tumor suppressor in human malignancies, and mutations of this gene are reported in human tumors such as endometrial, pancreatic, and colorectal cancers, melanoma, and leukemia (Strohmaier et al., 2001; Spruck et al., 2002; Calhoun et al., 2003; Rajagopalan et al., 2004; Kemp et al., 2005;

Akhoondi et al., 2007; Balakrishnan et al., 2007; Malyukova et al., 2007).

No mutations of *FBXW7* have thus far been reported in brain tumors, but an allelotyping study of glioblastoma showed frequent LOH at 4q31, the locus for *FBXW7* (Hu et al., 2003). We have reported that mRNA expression of *FBXW7*, particularly of the β -form, was highly suppressed in all the human glioma cell lines analyzed (A172, U87MG, U251MG, U373MG), and contrastive enhanced expressions of CCNE1, MYC, and AURKA were observed by Western blotting (Gu et al., 2007). Furthermore, the *FBXW7* β -form is reported to be expressed as the predominant form in the brain (Strohmaier et al., 2001). Taking the following fact that particular silencing of the *FBXW7* β -form is coupled with up-regulation of CCNE1, MYC, and AURKA in all the four glioma cell lines into consideration, our data strongly suggest that the inactivation of the *FBXW7* β -form plays an important role in the pathogenesis of gliomas. In the present study, we attempted to determine the silencing mechanisms of the *FBXW7* β -form.

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MATERIALS AND METHODS

Cell culture and normal cells from brain and peripheral blood Four human glioma cell lines, A172, U87MG, U251MG, and U373MG, were obtained and cultured as previously described (Gu et al., 2007). Non-cancerous cerebrum tissue from a Japanese patient was obtained at autopsy in Tohoku University Hospital (Sendai, Miyagi, Japan). Peripheral blood cells were obtained from the healthy volunteers. The specimens were collected under written informed consent, and the study was approved by the Ethics Committee of Tohoku

University School of Medicine.

Treatments with 5-azacytidine and trichostatin A Treatments with 5-azacytidine and trichostatin A were performed according to methods described previously (Xu et al., 2005). In brief, $1-2 \times 10^5$ cells were seeded in 10 cm culture dishes (in diameter) and maintained in the culture medium containing 5-azacytidine (Sigma, St Lois, MO) at 2 μ M or 5 μ M for 5 days. At the last medium replacement, trichostatin A (Wako, Osaka, Japan) was added at 300 nM optionally, and the cells were harvested 24 hours later for further investigation.

Table 1. Nucleotide Sequences of the Primers and Probes

	Forward (5' to 3')	Reverse (5' to 3')	Size of product (bp)	Anneal. temp. T(°C)
RT-PCR				
^a E1α-2F/E4-2R	GTGGACCTGCCCGTTCACCA	TGAAATGAAGTCTCGTTGAAACT	399	55
^a E1β-2F/E4-2R	TATGGGTTTCTACGGCACATT	TGAAATGAAGTCTCGTTGAAACT	385	55
^a E1γ-2F/E4-2R	CCCAAGGCCTCCCTTTTTGT	TGAAATGAAGTCTCGTTGAAACT	374	55
^b B2M-F/R	TTTCAGCAAGGACTGGTCTTT	CCAAATGCGGCATCTTCAAAC	171	55
Quantitative RT-PCR				
<i>FBXW7</i> β-F/R	AGTACCACTGGGCTTGTAAC	CTCTGGTCCACTCCAGCTCT	160	60
<i>FBXW7</i> β probe	ACGCCGAATTACATCTGTCCAGCCA			
^b B2M-F/R	TTTCAGCAAGGACTGGTCTTT	CCAAATGCGGCATCTTCAAAC	171	60
^b B2M probe	CTGAAAAAGATGAGTATGCCTGCCGTGTG			
Genomic bisulfite-modification specific PCR and sequencing for <i>FBXW7</i>				
E1β-BF/-BR	TAAAATGTTTTGAAAAGATTTATTTG	TATTTTTCCCTTCAATAATTCTATA	621	60

Nucleotide sequences of the primers for ^aFBXW7 and ^bB2M as well as ^bB2M probe have previously been reported in Gu et al. (2007) and Ogawa et al. (2006), respectively.

Fig. 1. Results of quantitative real time RT-PCR of *FBXW7* β -form after treatments with 5-azacytidine (5-aza) and trichostatin A (TSA) in gliomas. The cells were treated with 5-azacytidine at different concentrations for 96 hours followed by trichostatin A treatment and 5-azacytidine for 24 hours, and the relative mRNA levels of *FBXW7* β -form were measured by the quantitative real time RT-PCR assay. In each case, results are shown as the changes relative to the no treatment cells, as indicated by the open box. Each experiment was performed in triplicate, and error bars denote the standard deviations.

DNA and RNA extraction, RT-PCR, and quantitative real time RT-PCR Total genomic DNAs and RNAs were extracted using a DNeasy Blood & Tissue Kit (50) and an RNeasy midi kit (QIAGEN, Tokyo, Japan),

respectively, following the manufacturer's protocols. First strand cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA) according to

the manufacturer’s protocol. RT-PCR was performed by the methods described previously (Mori et al., 1997). Quantitative real time RT-PCR was performed as described (Xu et al., 2005), using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Specific primers and probes for the *FBXW7* β -form were designed by using the Primer Express software (Applied Biosystems, Foster City, CA). Primers and the FAM-TAMURA dual-labeled probes were purchased from Sigma Genosys (Ishikari, Japan). Expression of the *B2M* (β 2 microglobulin) gene was monitored as an internal control. The nucleotide sequences of the primers and the probes, and the conditions for RT-PCR are summarized in Table 1. Amplifications were carried out in 25 μ l of the reaction mixture containing 5 μ l of cDNA samples using a program comprised of 2 min. at 50°C, then 15 min. at 95°C, and then 40 cycles, each consisting of 15 sec. at 95°C and 1 min. at 60°C. The expression ratio of *FBXW7/B2M* was calculated and used. Each experimental reaction was performed in triplicate.

Genomic sodium bisulfite sequencing analysis
Genomic sodium bisulfite sequencing analysis was performed by methods described previously (Xu et al., 2005) using each 1 μ g of genomic DNA and a CpGenome™ DNA Modification Kit (Chemicon, Temecula, CA). Each modified DNA was used as a template for PCR amplifications, and their nucleotide sequences were determined by the method described previously (Sakurada et al., 1997). Nucleotide sequences of the primers and PCR conditions are summarized in Table 1.

Statistics All experiments were performed at least

three times independently. Statistical calculation was done using Statview software (SAS Institute Inc., Cary, NC).

RESULTS

Elucidation of *FBXW7* β -form expression after 5-azacytidine and trichostatin A treatments We previously demonstrated that the *FBXW7* β -form is specifically suppressed in all the glioma cell lines analyzed, but no genetic alterations were found in the entire exonic regions of the *FBXW7* gene (Gu et al., 2007). In this study, we used the same four glioma cell lines, A172, U87MG, U251MG, and U373MG. To determine whether the decreased expression level of *FBXW7* β -form can be attributed to an epigenetic modification, we treated all four cell lines with 5-azacytidine (5-AZA) and trichostatin A (TSA); there were no changes in cell growth (data not shown). We further performed quantitative real-time PCR analysis to determine the intrinsic expression levels of the *FBXW7* β -form. As shown in Fig. 1, no obvious increases were observed in any of the analyzed cells except for U373MG with the statistically significant upregulation at 5 μ M of 5-AZA with TSA. It was very likely that epigenetic mechanisms are not playing a major role in the transcriptional suppression of *FBXW7* β -form in the majority of these glioma cells, but U373MG needs further investigation of its methylation status.

Determination of the methylation status in the promoter region of *FBXW7* β -form Next we tried to determine the methylation status in the putative promoter and the 5’ region in exon 1 in the four cell lines using the

Fig. 3. Results of semi-quantitative RT-PCR analysis of three isoforms of *FBXW7* in normal peripheral blood cells and a cerebrum sample. Primer sets E1 α -2F/E4-2R, E1 β -2F/E4-2R, and E1 γ -2F/E4-2R were used for specific amplification of the *FBXW7* α -, β - and γ -forms, respectively. *B2M* was used as the internal control. cDNA from the normal cerebrum was used as the positive control, and water (DW) was used as the negative control.

sodium bisulfite-modification methods followed by sequencing analysis. The nucleotide sequence of this region is shown in Fig. 2A, and a representative result of sequencing analysis in U373MG is shown in Fig. 2B. We did not find any methylated sites in any of the analyzed cell lines. As controls, we examined one normal non-cancerous sample of cerebrum tissue from a patient at autopsy and 11 peripheral blood DNA samples (6 males and 5 females). The CpG sites in the putative promoter region and exon 1 of *FBXW7* β -form were partially methylated in the cerebrum and highly methylated in all the peripheral blood cells (see Fig. 2B); the cerebrum may have been affected by some contaminated peripheral blood cells. We further determined the nucleotide sequences in the putative promoter region to elucidate the involvement of structural alteration in the four cell lines, but no genetic alterations were found (data not shown).

Determination of the expression of *FBXW7* isoforms To investigate whether or not hypermethylation actually represses the expression of *FBXW7* β -form in human peripheral blood cells, the mRNA level of each isoform was analyzed by semi-quantitative RT-PCR. Results are shown in Fig. 3; suppressed expression of only the *FBXW7* β -form was observed in the peripheral blood cells; it is probable that hypermethylation repressed the expression.

DISCUSSION

FBXW7 functions as an ubiquitin ligase that can degrade several important cell cycle accelerators, its mutations or reduced expressions will associate with human tumors (Welcker and Clurman, 2008). Our previous studies have reported that mRNA expression of *FBXW7*, particularly the β -form, was highly suppressed in all the human glioma cell lines analyzed, although no genetic alterations were observed in the *FBXW7* gene (Gu et al., 2007). The β -isoform has been reported to be the major transcript in human brain (Strohmaier et al., 2001), and we observed the dominant expression of this form in human cerebrum, as shown in Fig. 3. Recently, highly reduced expression of *FBXW7* was observed but the *FBXW7* allele retained in primary glioblastomas (Hagedorn et al., 2007). DNA methylation and histone modification play physiological roles in the regulation of gene expression during development and in inactivation of the X chromosome (Barlow, 1995; Goto and Monk, 1998), and imbalances of these processes also seem to be important steps in malignant transformation (Baylin, 2005). In our present study analyzing the mechanisms of suppressed expression of *FBXW7* β -form, the glioma cells did not respond to reagents modifying the DNA methylation or to histone deacetylation. Results of nucleotide sequencing analyses after bisulfite modifica-

tion also supported the idea that such epigenetic mechanisms are not the major cause of suppression of the *FBXW7* β -form in glioma cells. Furthermore, no structural alterations were found in this gene. Some other mechanisms such as expressional or functional alteration of the associating transcription factors are probably involved in this expressional suppression.

As shown in Fig. 2A, binding sites for some transcription factors are located in the putative transcriptional regulatory region. However, most of these sites do not harbor CpG dinucleotide sequences. The *FBXW7* β -form has been reported as a direct target of TP53 (Kimura et al., 2003), and a TP53 consensus binding site is included in exon 1 of the *FBXW7* β -form (see Fig. 2A). U251MG and U373MG harbor the same R273H mutations in TP53 (Van Meir et al., 1994), but A172 and U87MG have wild type TP53 (Van Meir et al., 1994; Jia et al., 1997); because mutation status of p53 was controversial in A172 (C242F by Gomez-Manzano et al. (1996) using A172 from ATCC and wild type TP53 by Fukami et al. (2004) using A172 from Institute of Development, Aging and Cancer, Tohoku University, the same as our cell line), the mutation status of p53 along with the STR pattern of our A172 was analyzed and found wild type p53 and identical STR pattern to the report from ATCC. Thus TP53 status alone cannot explain the suppressed expression in these glioma cell lines. Further studies are necessary to uncover the mechanisms underlying the biological significance of the underexpression of *FBXW7* in glioma cell lines.

It is interesting that the *FBXW7* β -form was hypermethylated and did not show expression in human normal peripheral blood cells. As the *FBXW7* α -form and γ -form expressed in human peripheral blood cells, it is probable that these forms function predominantly in hematopoietic cells. Mutational inactivation of *FBXW7* is also found in some hematologic tumors (Akhoondi et al., 2007; Malyukova et al., 2007; Onoyama et al., 2007; Thompson et al., 2007), and recent review on the T-cell leukemia and lymphoma indicated the involvement of inactivation of *FBXW7* in tumorigenesis (Aifantis et al., 2008). Our recent results suggested an association between methylation of *FBXW7* β -form and progression of thymoma (Gu et al., MS in preparation). These accumulating data suggest that impairment of *FBXW7* is one of the critical events in the course of hematologic carcinogenesis.

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