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The *FBXW7 β-form* is suppressed in human glioma cells

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Abstract

FBXW7 (F-box and WD40 domain protein 7) is an F-box protein with 7 tandem WDs (tryptophan–aspartic acid) that functions as a phosphoepitope-specific substrate recognition component of SCF (Skp1–Cul1–F-box protein) ubiquitin ligases and catalyzes the ubiquitination of proteins promoting cell proliferation, such as CCNE1, MYC, AURKA, NOTCH1, and JUN, which are frequently activated in a wide range of human cancers. *FBXW7* is a candidate tumor suppressor, and mutations have been reported in some human tumors. In this study, we analyzed 84 human tumor cell lines in search for genetic alterations of *FBXW7*, as well as mRNA and protein expressional changes, and compared them with expression levels of the CCNE1, MYC, and AURKA proteins. We found a novel nonsense mutation in a colon cancer cell line SCC and confirmed the missense mutations in SKOV3, an ovarian cancer cell line, and LoVo, a colon cancer cell line. Moreover, suppressed expression of FBXW7 accompanied by activation of the target proteins were observed in ovarian, colon, endometrial, gastric, and prostate cancers. It is notable that highly suppressed mRNA expression of the *FBXW7 β-form* was found in all the human glioma cell lines analyzed; enhanced expressions of CCNE1, MYC, and AURKA were observed in these cells. Our present results imply that FBXW7 plays a pivotal role in many tissues by controlling the amount of cell cycle promoter proteins and that dysfunction of this protein is one of the essential steps in carcinogenesis in multiple organs.

Keywords: Mutations; FBXW7; CCNE1; MYC; AURKA; SCC; Glioma

FBXW7 (F-box and WD-40 domain protein 7, also known as FBW7, FBXW6, FBX30, CDC4, SEL-10, SEL10, AGO, and FLJ11071) is a member protein of the F-box containing components of the SCF (Skp1–Cul1–F-box) ubiquitin ligase complexes, which play an important role in the degradation of proteins regulating G1–S cell cycle progression [1]. In addition to the three invariable components (Skp1, Cul1, and Rbx1) comprising the SCF complexes, one of the variable F-box proteins is always included for substrate specificity [1–3]. Among the F-box proteins thus far identified, Skp2 (S phase kinase-associated protein 2) and FBXW7 have been well characterized and shown to control an abundance of proteins important in cell cycle regulation [4]. FBXW7 has been identified as a negative regulator of CCNE1 [5], MYC [6], AURKA [7], NOTCH1 [8], and JUN [9], all of which appear to function as cell cycle promoters and oncogenic proteins if up-regulated.

The *FBXW7* gene encodes three protein isoforms, the FBXW7 α-, β-, and γ-form, which are translated from mRNAs transcribed from distinct 5′ exons with individual and unique promoters and joined with 10 shared exons [10]. This gene maps in the 4q31 region, which is reported to be deleted in several cancers, including glioblastoma multiforme, nasopharyngeal carcinoma, and small-cell carcinoma of the breast [11]. Loss of *FBXW7* in cancer may thus have profound effects on the pathways that govern cell division, differentiation, apoptosis, and cell growth. It is also reported that *FBXW7* is mutated in cancer cell lines of the breast [5] and ovary [12], as well as primary tumors of the pancreas [13], endometrium [14], colorectum [15], and ovary [16]. Furthermore, linkage analysis suggested the existence of a gene responsible for familial pancreatic cancer [17]. It is further reported that inactivation of *FBXW7* resulted in chromosomal instability [18]. For these

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many reasons, it is of great interest to explore abnormalities of the *FBXW7* gene in human cancers and to compare their interactions with cell cycle promoter proteins such as CCNE1, MYC, and AURKA.

Materials and methods

Cell lines and normal tissues. A total of 84 human cancer cell lines were analyzed in this study; 21 lung cancer cell lines (1–87, 11–18, 86–2, ABC-1, H1299, LCSC#1, LCSC#2, LK79, LK87, Lu65, Lu99A, Lu99B, RERF-LC-MS, RERF-LC-OK, Sato T, S-2, SBC3, Sq-1, Sq19, VMRC-LCD, VMRC-LCP), 28 pancreatic cancer cell lines (PK-1, PK-8, PK-9, PK-12, PK-14, PK-16, PK-36, PK-45 P, PK-45 H, PK-47, PK-59, PK-65, PK-66, PK-67, PAN03JCK, PAN07JCK, PAN08JCK, PAN09JCK, PCI-6, PCI-19, PCI-24, PCI-35, PCI-43, PCI-55, PCI-64, PCI-66, MIAPaCa2, Su86.86), 10 colon cancer cell lines (DLD-1, HCT116, HCT15, Colo205, CoLoTc, LoVo, LS174T, SCC, SW480, WiDrTc), seven ovarian cancer cell lines (JHOC5, JHOS2, OVK18, OVK18#102, SKOV3, TOV112D, TOV21G), six endometrial cancer cell lines (AN3CA, HEC1-A, Ishikawa 3H12, KLE, RL95-2, SK-UT-1B), five gastric cancer cell lines (AZ521, MKN7, MKN28, SNU-5, SNU-668), four glioma cell lines (A172, U87MG, U251MG, U373MG), one prostate cancer cell line (LNCaP), one osteosarcoma cell line (U2OS), and one breast cancer cell line (MCF7). Most of these cell lines have been analyzed in our previous studies [19–29]. The human colon cancer cell line SCC was obtained from Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). The human bronchial epithelial cell line Beas2B was obtained from the American Type Culture Collection (Manassas, VA), and the immortalized normal human pancreatic ductal cell HPDE [30] was kindly provided by Dr. Ming S. Tsao. Each of the cell lines was cultured in the medium recommendations by its supplier and supplemented with 10% fetal bovine serum (Invitrogen, San Diego, CA) under an atmosphere of 5% CO_2 with adequate humidity at 37 °C. The 5-azacytidine with and without trichostatin A treatments were done by the methods described previously [31]. The four normal surgically resected human tissues (ovary, uterus, stomach, and colon) and the two non-cancerous tissues (cerebrum and prostate) from autopsy patients, all of which were used as controls, were obtained at Tohoku University Hospital (Sendai, Miyagi, Japan). All the patients were Japanese, and the specimens were collected under written informed consent. This study was approved by the Ethics Committee of Tohoku University School of Medicine.

DNA extraction and mutation search. Genomic DNAs were extracted using Nucleon™ BACC2 Genomic DNA Extraction Kit for Blood & Cell Cultures (Amersham Biosciences, Little Chalfont, UK) following the manufacturer's protocol. The purified DNAs from the tumor cell lines were PCR amplified for the whole exons and their intron–exon boundaries of *FBXW7* by methods described previously [32] with the GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA). Primer pairs for individual exons and PCR conditions are listed in Table 1. Amplified DNAs were run on 2% agarose gels, and the bands were cut out and purified by electro-elution followed by ethanol precipitation. The nucleotide sequences were determined by methods described previously [33] using the Hi Di Formamide (Applied Biosystems) and ABI PRISM3100 Genetic Analyzer (Applied Biosystems).

RNA extraction and RT-PCR. Total RNAs were extracted from the harvested cells and normal tissues using the RNeasy midi kit (QIAGEN, Tokyo, Japan) according to the supplier's instructions. Each purified RNA was dissolved in RNase-free water, and its concentration was measured by optical absorbance at 260 nm. Random primed first strand cDNA was synthesized using an aliquot of 10 μg of total RNA and SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) by the methods described previously [34]. The nucleotide sequences of the primers and the conditions for RT-PCR are summarized in Table 1. Expression of the *B2M* (β2 microglobulin) gene was monitored as the internal control.

Western blotting analyses. Western blotting analyses were performed as described previously [35]. Rabbit anti-human FBXW7 α-form polyclonal antibody (PAB-10563) from Orbigen (San Diego, CA), mouse anti-human Cyclin E monoclonal antibody (sc-247) and rabbit anti-human MYC polyclonal antibody (sc-764) from Santa Cruz Biotechnology (Santa Cruz, CA), rabbit anti-human Aurora kinase A polyclonal antibody from Transgenic (Kumamoto, Japan), mouse anti-human β-actin monoclonal antibody from Sigma (St. Louis, MO), and horseradish peroxidase-conjugated with anti-mouse or anti-rabbit immunoglobulin antibodies from Amersham Biosciences (Piscataway, NJ) were used following the instructions by the suppliers. Detection was performed using an ECL detection kit (Amersham Biosciences) according to the supplier's instructions.

Table 1
Nucleotide sequences of the primers and probes

	Primer sequence (5′ to 3′)		Size of product (bp)	Anneal. temp. (°C)
	Forward	Reverse		
Genomic DNA				
Exon 1α	CAAGGTCCAAGAAGTAGCAA	TAGATATGTAAAGTTTCTCAGG	582	55
Exon 1β	GAATCTGTTCAGTCAAGGCTT	CACAGTCTTTCCGTTATTTGC	548	55
Exon 1γ	TTAGTGGTTAGAGCTTTTAGTAA	TCTTTTCACTAAAAGAGGCCAA	293	55
Exon 2	TTAACCATGCTGACTCAAGATT	CAGTCAACCGTACTAGTAACA	266	60
Exon 3	TGCCAGATCATCATTCTTTGTA	TTACGGATGAATAATCCCTTATT	328	55
Exon 4	CTGTAATTTGGGACATCTGTT	CCATAATTAGCATGACAATGTT	280	55
Exon 5	GTATCTCATCCTGTGGAGAATA	CGGCTCATCTGAATGTGTAGA	352	55
Exon 6	CTGTTATTATTGGTGAAGGCAA	TAGGTACTAACACTGATTAACG	238	55
Exon 7	GATAGACTACAAATTACTGTTC	CAGTTGCTACTTGCAATGATA	224	55
Exon 8	GGGATCATTTTATACGGATGTA	TTAAGAGCACACTGTCACTATT	431	55
Exon 9	CCTTGACTAAATCTACCATGTT	GACTGTACTGGATCAGCAATT	320	55
Exon 10	CTTTCCTACTAGGATTAAGGT	TGAACAAAACGAAAGGTGAGT	320	55
Exon 11	TGTAACTAACTCATAGCCATTAT	GGGAGTATATCGTCTACACAA	377	55
RT-PCR				
E1α-2F/E4-2R	GTGGACCTGCCCGTTCACCA	TGAAATGAAGTCTCGTTGAAACT	399	55
E1β-2F/E4-2R	TATGGGTTTCTACGGCACATT	TGAAATGAAGTCTCGTTGAAACT	385	55
E1γ-2F/E4-2R	CCCAAGGCCTCCCTTTTTGT	TGAAATGAAGTCTCGTTGAAACT	374	55
E3-2F/E4-2R	AGTACCACTGGGCTTGTACC	TGAAATGAAGTCTCGTTGAAACT	264	55
E8-2F/E10-2R	TGTGCGTTGTATGCATCTTCA	GATCCACTCACCACATGGAT	285	55
B2M-F/B2M-R	TTTCAGCAAGGACTGGTCTTT	CCAAATGCGGCATCTTCAAAC	171	55