p53-Altered FBXW7 Expression Determines Poor Prognosis in Gastric Cancer Cases

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Abstract

A molecular target associated with the progression of gastric cancer has not yet been uncovered. FBXW7 is a tumor suppressor gene transcriptionally controlled by p53 that plays a role in the regulation of cell cycle exit and reentry via c-Myc degradation. Few studies have addressed the clinical significance of FBXW7 expression in gastric cancer. Low expression levels of FBXW7 in primary gastric cancer contributed to malignant potential, such as lymph node metastasis (P = 0.0012), tumor size (P = 0.0003), and poor prognosis (P = 0.018). In comparison with 52 cases of gastric cancer without the p53 mutation, 29 cases with the mutation exhibited lower expression levels of FBXW7 (P = 0.0034), revealing a significant relationship between p53 mutation and FBXW7 expression. Furthermore, we found that gastric cancer patients who had low FBXW7 expression levels and p53 mutation had a distinctively poor prognosis in comparison with other subgroups (P = 0.0033). In conclusion, we showed a role for p53 in the transcriptional regulation of FBXW7 expression in clinical gastric cancer cases and showed that disruption of both p53 and FBXW7 contributes to poor prognosis. [Cancer Res 2009;69(9):3788–94]

Introduction

FBXW7 is a F-box protein subunit of a SCF-type ubiquitin ligase complex that induces the degradation of positive cell cycle regulators (oncoproteins) such as c-Myc, cyclin E, c-Jun, and Notch. Therefore, FBXW7 and the associated molecules have been focused on as one of the new carcinoma control structures (1,2). In particular, FBXW7 induces cell cycle exit (G0 phase) via c-Myc degradation, so the altered expression of FBXW7 is considered one of the major causes of carcinogenesis or carcinoma development (2–4). Because FBXW7 also participates in cell cycle exit to, and the reentry from, G0 (5–7), it is a candidate molecular therapeutic target in intractable carcinoma cases that are firmly resistant to combined modality therapies (5,8).

Mao and colleagues reported that epithelial tumors are not established in p53−/− mice, whereas p53+/− mice form epithelial tumors with altered FBXW7 expression. FBXW7 works downstream of p53, both of these cell cycle regulator genes, are critical for carcinogenesis of epithelial tissues (3).

Recently, Onoyama and colleagues reported that mice carrying a FBXW7 T-cell conditional knockout eventually developed thymic lymphomas following thymomas. FBXW7 and p53 double-knockout mice developed thymic lymphomas more frequently than other subgroups of knockout mice, such as wild-type, p53−/−, and FBXW7 conditional knockout mice. Therefore, their study clearly showed the consecutive roles of p53 and FBXW7 in the carcinogenesis of solid tumors in vivo. Moreover, a comparison of four groups classified according to FBXW7 and p53 status revealed a worse prognosis for double inactivation mice than in the other subgroups (6). It is unknown if identical findings were observed during previous in vivo studies of human cancer cases.

The clinical significance of FBXW7 in human solid cancers has been diversely reported. FBXW7 mutation rates in cholangiocarcinomas, T-cell acute lymphocytic leukemia, endometrial carcinoma, and colorectal cancer were reported as 35%, 31%, 9%, and 9%, respectively (2,4,9,10). Also, FBXW7 low expression in glioma tissues reportedly produces a poor prognosis (11,12). Lee and colleagues reported that the FBXW7 mutation rate in clinical gastric cancer tissues of 3.7% to 6% did not differ in early or progressive gastric cancer (4,13). However, few studies are available on the connection between FBXW7 expression level and poor prognoses in gastric cancer.

This study details (a) the magnitude of the effect of altered FBXW7 expression on prognosis determination in gastric cancer cases; (b) the significance of both FBXW7 expression and p53 mutation status on clinical gastric cancer cases, which was compared with previous in vivo reports; and (c) how the coexistence of the p53 mutation and low expression of FBXW7 in clinical samples determines malignant potential and a poorer prognosis for gastric cancer patients.

Materials and Methods

Clinical samples and cell lines. One hundred gastric cancer samples and paired noncancerous tissues were obtained during surgery and used after obtaining informed consent. All patients underwent resection of the primary tumor at Kyushu University Hospital at Beppu and affiliated hospitals between 1992 and 2000. Resected cancer tissues and paired noncancerous tissues were immediately cut and embedded in Tissue-Tek OCT medium (Sakura), frozen in liquid nitrogen, and kept at −80°C until RNA and DNA extraction. Following isolation of RNA and DNA, cDNA was synthesized from 8.0 μg total RNA as described previously (14).

The human gastric cancer cell line AZ521 was provided by the Cell Resource Center of Biomedical Research, Institute of Development, Aging
and Cancer, Tohoku University. This cell line was maintained in RPMI 1640 containing 10% fetal bovine serum with 100 units/mL penicillin and 100 units/mL streptomycin and cultured in a humidified 5% CO2 incubator at 37°C.

Real-time quantitative reverse transcription-PCR. FBXW7-specific oligonucleotide primers were designed to amplify a 249-bp PCR product encoding the common region among three FBXW7 isoforms. The following primers were used: FBXW7 sense primer 5′-AAAGAAGTTG-TAGCCGGTTCTCG-3′ and antisense primer 5′-CCACATGGATACCAT-CAAACCTG-3′ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 270 bp) sense primer 5′-GCTAACGGATTGGTCTGTATT-3′ and antisense primer 5′-AGCTCTTGGTGCCAGTGAT-3′. These primers spanned more than two exons to avoid amplification of contaminating genomic DNA. PCR amplification for quantification of FBXW7 and GAPDH mRNA in clinical samples was done in the LightCycler system (Roche Applied Science) using the LightCycler-FastStart DNA Master SYBR Green I Kit (Roche Applied Science). Primers were used for 10 s, followed by 40 cycles of denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 62°C [60°C for GAPDH] 10 s, and elongation at 72°C [65°C for GAPDH] 10 s. Melting curve analysis was done to distinguish specific products from nonspecific products and primer dimmers. The relative expression levels of FBXW7 were obtained by normalizing the amount of FBXW7 mRNA divided by that of GAPDH mRNA as an endogenous control in each sample.

FBXW7 RNA interference. FBXW7-specific siRNA (Silencer Predesigned siRNA: sense GCCAGAUAUGAUACUACTT and antisense GUUAUUAUCCAUUCUGGCUGTTG) and Silencer Predesigned siRNA2: sense CCAUUAAUAGGGCAUAUCUCLTT and antisense GAAGUAGGCAUAAAGGTG and negative control siRNA (Silencer Negative Control 1 siRNA) were purchased from Ambion. Lipofectamine RNA interference MAX (Invitrogen) and FBXW7-specific siRNA were then added in 6-well flat-bottomed microtiter plates. After incubation, the AZS21 cell line was seeded at 1.5 × 10^4 per well in a volume of 2 mL in 6-well flat-bottomed microtiter plates and incubated in a humidified atmosphere (37°C and 5% CO2). The RNA interference assay was done after a 24-h incubation.

Immunoblot analysis. Total protein was extracted from AZS21 after FBXW7 RNA interference. Aliquots of total protein (35 μg) were electrophoresed in 7.5% concentrated REDELS G (Bio-Rad Laboratories), c-Myc, cyclin E, and p53 proteins were detected using anti-c-Myc (N262), anti-cyclin E (M20), and anti-p53 (Pab240; all obtained from Santa Cruz Biotechnology) diluted 1:500, 1:100, and 1:100, respectively. These proteins were normalized to the level of β-actin protein (Cytoskeleton) diluted 1:100. Western blot analysis was done as described previously (16).

Figure 1. Clinical significance of FBXW7 mRNA expression in clinical samples. A, FBXW7 mRNA expression in cancer (T) and noncancerous (N) tissues from gastric cancer patients by real-time reverse transcription-PCR (n = 100), FBXW7 (T; n = 100), FBXW7 mRNA (T)/GAPDH mRNA (T); FBXW7 (N; n = 100), FBXW7 mRNA (N)/GAPDH mRNA (N). Horizontal lines, mean. B, Kaplan-Meier overall survival curves of gastric cancer patients according to the level of FBXW7 mRNA expression. The high FBXW7 expression group (n = 31), FBXW7 (T)/FBXW7 (N) ≥ 1.0; low FBXW7 expression group (n = 69), FBXW7 (T)/FBXW7 (N) < 1.0. Enhanced chemiluminescence detection reagents (Amersham Biosciences) were used to detect antigen-antibody reactions.

In vitro proliferation assay. Proliferation was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Roche Diagnostics). After a 24-h incubation following siRNA addition, cells were cultured further for 0 to 72 h and the absorbance of the samples was measured as described previously (17).

p53 and FBXW7 sequence. Among 100 gastric cancer samples in which FBXW7 mRNA levels were measured, p53 was sequenced in 81 genomic DNA samples. Similarly, 80 paired cDNA samples were subjected to FBXW7 mutational analysis.

The 81 genomic DNA samples were used as templates to PCR amplify exons 4 to 9 of the p53 gene with primers derived from intronic sequences (Supplementary Table S1). The PCR was done with AmpliTaq Gold DNA Polymerase (Applied Biosystems). Likewise, the FBXW7 (α, β, and γ) sequence was amplified using cDNA from 80 gastric cancer samples with KOD-FX DNA polymerase (TOYOBO) and sequencing primers (Supplementary Table S1). These PCR products were electrophoresed on 1% agarose gels containing ethidium bromide and purified with ethanol precipitation. Purified PCR products were sequenced using a Big-Dye Terminator version 1.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI3100 sequencer (Applied Biosystems).

Statistical analysis. Differences between two groups were estimated with Student’s t test, χ2 analysis, and ANOVA. Overall survival curves were plotted according to the Kaplan-Meier method, with the log-rank test applied for comparison. Survival was measured from the day of the surgery. Data for FBXW7 mRNA expression levels in three groups were analyzed with ANOVA. When the results of the ANOVA were significant, Tukey’s multiple comparison tests were used to assess differences in FBXW7 mRNA expression levels among each group. All differences were statistically significant at the level of P < 0.05 and a tendency was indicated at the level of P < 0.1. Statistical analyses were done using the JMP 5 for Windows software package (SAS Institute).

Results

Clinical significance of FBXW7 mRNA expression in gastric cancer cases. The expression levels of FBXW7 mRNA in cancerous tissues (n = 100) and paired noncancerous tissues (n = 100) of the gastric cancer patients were examined by real-time reverse transcription-PCR. These data were corrected for GAPDH mRNA levels. FBXW7 mRNA expression levels in cancer
tissues (mean ± SD, 1.41 ± 1.51) were lower than those in noncancerous tissues (2.37 ± 2.3). A significant difference in mRNA mean expression level was found between cancerous and noncancerous tissues (P = 0.0007; Fig. 1A). Immunostaining of FBXW7 was done to confirm the correlation between mRNA and FBXW7 protein. Fifteen gastric cancer samples were divided into two groups according to FBXW7 protein level (high or low). The expression of FBXW7 mRNA in each group was examined and compared with protein expression levels. The high FBXW7 protein group (n = 7) showed high FBXW7 mRNA expression levels in comparison with the low FBXW7 protein group (n = 8; P = 0.0013; Supplementary Fig. S1).

In the overall survival curve (Fig. 1B), patients in the low FBXW7 expression group (n = 69; cancer/noncancerous tissues < 1.0) had a significantly poorer prognosis than those in the high FBXW7 expression group (n = 31; cancer/noncancerous tissues ≥ 1.0; P = 0.018). However, there was no relationship between FBXW7 expression and clinical stage progression (Supplementary Fig. S2). Multivariate analysis revealed that the FBXW7 mRNA expression level in cancer is an independent predictor of lymph node metastasis (Supplementary Table S2A and B).

Clinicopathologic factors were significantly different in the low FBXW7 expression group (n = 69). There was more progressive tumor size, lymph node metastasis, venous invasion, peritoneal dissemination, and clinical staging compared with the high FBXW7 expression group (n = 31; P < 0.05). However, no significant differences were observed regarding age, gender, histology, lymphatic invasion, and liver metastasis (Table 1).

Expression of the FBXW7 isoform and prognosis in gastric cancer cases. In several in vivo studies, mouse Fbxw7 has three isoforms (α, β, and γ). The α isoform is expressed in most tissues, the β isoform is found in the brain and testis, and the γ isoform is in the heart and muscle (5). We confirmed the distribution of FBXW7 expression in a human panel before searching for FBXW7 mutations in gastric cancer cases. We found a similar distribution of FBXW7 mRNA expression between the human panel and laboratory mice (Supplementary Fig. S3). Fbxw7 γ controls the nuclear level of c-Myc and cell size and is restricted to muscle cells, which is larger than other cells (5, 18). It has been suggested that Fbxw7 γ contributes to muscle differentiation through regulation of c-Myc. Therefore, the expression level of FBXW7 γ in the heart might be very high to regulate heart muscle differentiation (Supplementary Fig. S3).

In addition, the association between overall FBXW7 expression and poor prognosis was more significant than between the expression of any individual isoform (Supplementary Fig. S4).

**FBXW7 and p53 mutation analysis.** We examined p53 mutations in 81 genomic DNA samples and FBXW7 mutations in 80 cDNA samples, the same paired samples that were used for the FBXW7 mRNA expression assay. Mutation analysis done with sequencing found p53 and FBXW7 mutation rates of 35.8% (29 of 81) and 8.8% (7 of 80), respectively (Fig. 2A and B; Supplementary Table S3; Supplementary Fig. S5).

Examination of the relationship between p53 mutation status and FBXW7 mRNA expression levels revealed that FBXW7 mRNA mean expression levels in the p53 mutation (+) group (n = 29; 1.07 ± 1.0) were lower than those in the p53 mutation (−) group (n = 52; 1.56 ± 1.79) and noncancerous tissues (n = 100; 2.36 ± 2.3). A significant difference was found between the p53 mutation (+) group and the other groups (Fig. 3). In addition, no difference was observed between the p53 mutation (−) gastric cancer tissues and noncancerous tissues.

Mean expression levels of FBXW7 in the FBXW7 mutation (+) group (n = 7) were not significantly different from those of the FBXW7 mutation (−) group (n = 73) and noncancerous tissues (n = 100). The presence of the FBXW7 mutation was not associated with poor prognosis or clinical stage in gastric cancer patients (Supplementary Fig. S5).

**FBXW7 RNA interference promotes proliferation in vitro.** Because FBXW7 mRNA suppression in cancer tissues is associated with poor prognosis, the protein levels of c-Myc and cyclin E, degradation targets of FBXW7, were examined to evaluate FBXW7 function in gastric cancer cells. FBXW7
suppression analysis was done with two different FBXW7 siRNA (siRNA1 or siRNA2) using gastric cancer cell line AZ521. FBXW7 suppression by siRNA was confirmed with quantitative reverse transcription-PCR in the control siRNA and FBXW7 siRNA groups. The level of FBXW7 mRNA was substantially reduced by 70% in FBXW7 siRNA1 (Fig. 4A).

Western blot analysis confirmed expression of c-Myc and cyclin E proteins degradation targets of FBXW7 in control siRNA and FBXW7 siRNA groups. The expression levels of c-Myc and cyclin E protein were enhanced in the FBXW7 gene in gastric cancer patients (n = 81). p53 mutation in gastric cancer patients was markedly decreased in comparison with the control siRNA group and parent cell line AZ521 (Fig. 4C).

**p53 mutation and FBXW7 expression are associated with poor prognosis in clinical gastric cancer patients.** FBXW7 mRNA expression was inhibited in p53 mutation (+) gastric cancer tissues, and the low FBXW7 expression patients had a significantly poorer prognosis than the high FBXW7 expression patients (Figs. 1B and 3).

Therefore, we divided 81 gastric cancer patients into four groups according to FBXW7 expression level and the state of the p53 mutation and examined the overall survival curve in these groups. The p53 mutation (+), FBXW7 low expression group (n = 24) had a significantly poorer prognosis than the other three groups (P = 0.0033; Fig. 5).

**Discussion**

In this study, we showed that FBXW7 mRNA expression in gastric cancer samples is markedly decreased in comparison with the corresponding noncancerous samples and that FBXW7 is a poor prognostic factor. There are three possible explanations. First, it is worth noting that FBXW7 expression is regulated by p53 in *in vitro* and *in vivo* experimental data (3, 5, 6, 19). For instance, Mao and colleagues reported that Fbxw7 mRNA expression was activated when p53 expression was induced by radiation, and baseline expression of Fbxw7 mRNA is suppressed in p53−/− mice. Moreover, they reported that a p53-binding site is present in a promoter region of the mouse Fbxw7 (3). In addition, Kimura and colleagues reported that FBXW7 β expression is enhanced when wild-type p53 is produced in a p53-mutated glioblastoma cell line (8). These reports strongly suggest that transcription of FBXW7 is regulated by p53 activity. Therefore, we focused on the regulation of FBXW7 expression by p53 in gastric cancer cases. In the current study, FBXW7 expression levels were decreased in most p53 mutation (+) gastric cancer samples (Fig. 3); only 6% (5 of 81) cases were FBXW7 high expression in p53 mutation (+; Fig. 5). Most of the p53 mutation (+) gastric cancer patients belonged to the FBXW7 low expression group. Therefore, we propose that FBXW7 mRNA expression is primarily regulated by the presence of the p53 mutation in clinical gastric cancer cases. It is worth noting that the reproducibility of this finding in *vivo* was clearly confirmed in human clinical cases. To determine which isoform of FBXW7 is regulated by p53 in *vivo*, we used p53 siRNA to suppress p53 expression in gastric cancer cell line AZ521. The expression levels of the three FBXW7 isoforms (α, β, and γ) were suppressed by p53 siRNA (Supplementary Fig. S6).

Second, we determined that FBXW7 is inactivated by mutation in the coding region. The average of FBXW7 mutation rate in several malignancies was ~6% (4). As for gastric cancer cases, Lee and colleagues reported the possibility of the presence of mutation, but the relationship of the FBXW7 mutation and prognosis was not elucidated (13). Therefore, we examined the sequence of the FBXW7 isoforms. The FBXW7 mutation rate, 8.8% (7 of 80), was similar to the 3.7% to 6% previously reported for gastric cancer (4, 13). Mutation hotspots are located in T-cell acute lymphocytic leukemia; however, they were not detected in the current study (13, 20, 21).
Third, chromosome 4q contains the FBXW7 gene. Approximately 30% of the gene is deleted in certain carcinomas, such as esophageal and gastric cancers. In particular, inactivation of tumor suppressor genes by the chromosome 4q deletion may be an important factor in colon carcinogenesis (22–24).

The reduction of FBXW7 expression is associated with the dysregulation of cyclin E and c-Myc, positive regulators of the cell cycle (2, 18, 25, 26). c-Myc is associated with cell growth and is recognized as an important factor in control of the G1 (G0) to S-phase transition (1, 6, 27, 28). Cyclin E expression is enhanced in various types of cancer, where it regulates cell cycle progression via Rb phosphorylation and contributes to genome instability (19, 29). Consistent with previously published reports, we showed that protein expression of c-Myc and cyclin E is enhanced when FBXW7 is suppressed in a gastric cancer cell line (Fig. 4). Immunohistochemical analysis of FBXW7 in clinical gastric cancer tissues revealed enhanced expression of Myc and cyclin E in FBXW7 low expression tissues (Supplementary Fig. S7A). Conversely, Myc and
cycin E expression levels were suppressed in tissues in which FBXW7 was overexpressed (Supplementary Fig. S7B), confirming the relationship between FBXW7 and target proteins in clinical gastric cancer tissues.

In addition, c-Myc accumulation induces p53-dependent apoptosis via MDM2 degradation (6, 30, 31). The inactivation of both FBXW7 and p53 promotes c-Myc accumulation and inhibits p53-dependent apoptosis by MDM2 activation. It probably means that the proliferation rate was increased in these cells.

The low FBXW7 expression group of gastric cancer patients showed progression of clinicopathologic factors and poor prognosis. All 4 cases of liver metastasis (100%, 4 of 4), 16 cases of peritoneal dissemination (94%, 16 of 17), and 24 cases of venous invasion (86%, 24 of 28) were classified as members of the FBXW7 low expression group (Table 1). Unfortunately, a significant correlation was not observed between the incidence of liver metastasis and FBXW7 expression because of an insufficient number of cases. However, other clinicopathologic findings indicated that FBXW7 contributes to hematogenous metastasis besides lymph node metastasis and peritoneal dissemination.

FBXW7 is a tumor suppressor. Considering tumor dormancy as one way to conquer malignancies, the introduction of FBXW7 may facilitate “tumor dormancy therapy.” Moreover, it was found that Myc inhibition triggers rapid regression of incipient and established lung tumors in vivo (32). Therefore, Myc degradation by FBXW7 may not only induce a state of tumor dormancy but also could have an antitumor effect.

As in a previous in vivo study, the simultaneous disruption of two cell cycle checkpoint genes, p53 and FBXW7, shortened the survival of mice with thymic lymphomas (6). It is notable that, even in the FBXW7 low expression group, the 5-year survival rate of p53 mutation (−) cases is 53%, but we found that it was 14% in the FBXW7 low expression/p53 mutation (−) group of clinical gastric cancer patients (Fig. 5). Both p53 and FBXW7 act to brake the cell cycle. Therefore, simultaneous disruption of these genes led to poor prognosis in clinical gastric cancer in comparison with inactivation of p53 or FBXW7 alone. Although p53 reportedly regulates FBXW7 expression, other mechanisms may be present. In the current study, most cases of p53 mutation (+) gastric cancer were in the low FBXW7 group and had poor prognosis (83%, 24 of 29). However, a few cases of p53 mutation (+) gastric cancer were in the high FBXW7 expression group (17%, 5 of 29) and had good prognosis in comparison with the p53 mutation (+)/low FBXW7 group. There were a few cancer cases with higher FBXW7 expression that was not regulated by p53. Therefore, the prognosis of p53 mutation (+) cases is not identical to that of p53 mutation (+)/FBXW7 low cases.

These results show that the status of FBXW7 and p53 is critical for prognosis determination in gastric cancer patients. This report is the first confirmation of the experimental mice data using clinical gastric cancer samples.

In conclusion, FBXW7 has recently attracted attention as a tumor suppressor gene that reduces important oncoproteins and related carcinogenesis and cell cycle progression. There are previous reports of in vitro and in vivo studies showing that p53 controls FBXW7 expression and that FBXW7 inactivation contributes to poor prognosis via genome instability and cell cycle progression. However, these findings had not been shown in clinical cancer samples. We have clarified that gastric cancer patients with inactivation of FBXW7 and p53 have a poorer prognosis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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