Genetic Alterations of the \textit{ATBF1} Gene in Gastric Cancer

Yong Gu Cho, Jae Hwi Song, Chang Jae Kim, Youn Soo Lee, Su Young Kim, Suk Woo Nam, Jung Young Lee, and Won Sang Park

\textbf{Abstract}  
\textbf{Purpose:} \(\alpha\)-Fetoprotein (AFP) – producing gastric cancers are aggressive tumors with venous and lymphatic invasion and hepatic metastasis. The goal of the present study was to investigate whether somatic changes of the AFP-negative regulator \textit{AT motif binding factor-1} (\textit{ATBF1}) gene are involved in the development or progression of gastric cancers and the production of AFP in gastric cancer cells.  
\textbf{Experimental Design:} We searched for genetic alterations of the \textit{ATBF1} gene by single-strand conformational polymorphism and sequencing methods as well as allelic loss analysis with the microsatellite markers D16S3066 and D16S3139. Immunohistochemistry for AFP expression in gastric cancer cells was also done.  
\textbf{Results:} In 81 sporadic gastric cancers, four mutations were detected in seven cases: one was a missense mutation and three were deletions; loss of heterozygosity at the \textit{ATBF1} locus was detected in 52.9\% of informative samples. Five of the eight cancers with AFP expression showed \textit{ATBF1} genetic alterations.  
\textbf{Conclusions:} These results suggest that genetic alteration of the \textit{ATBF1} gene may contribute to the aggressive nature of gastric cancers and the production of AFP in gastric cancer cells.

Gastric cancer occurs with a high incidence in Asia and is one of the leading causes of cancer deaths worldwide (1). In Korea, it accounts for an estimated 20.7\% of all malignancies, 24.2\% in males and 16.2\% in females (2). Therefore, gastric cancer remains a significant health burden worldwide. The precise molecular mechanism underlying gastric carcinogenesis is not fully understood.

\(\alpha\)-Fetoprotein (AFP) is a major plasma protein synthesized by the fetal liver, by yolk sac cells, and by some fetal gastrointestinal cells (3). AFP is not usually present in normal adult organs but can be found during the course of liver regeneration induced by hepatectomy (4) and in some adult cancer cells, such as hepatocellular carcinoma cells, yolk sac tumor cells, and gastric cancer cells (5–7). Among all cases of gastric cancer, AFP-producing gastric cancer accounts for 2\% to 28.5\% of cases (8–11). The AFP-producing gastric cancers show high proliferative activity and are recognized as tumors that have an extremely poor prognosis with a 5-year survival rate of \(<10\%\) (9, 10). The monitoring of AFP blood levels is used as an important diagnostic marker; however, the molecular mechanisms involved in AFP gene regulation remain unclear.

Recently, it has been reported that the hepatocyte nuclear factor-1 stimulates AFP transcription, whereas the AT motif binding factor-1 (ATBF1) was found to be a transcription suppressor (12–14). The ATBF1 has been characterized as a very large transcription factor; it was originally identified as a negative regulator of the AFP gene in studies where the inhibition of the activity of the AFP enhancer and promoter was observed in competition with hepatocyte nuclear factor-1 for a common binding site (13, 15). ATBF1 inhibits cell proliferation via transcriptional down-regulation of the Myb oncoprotein and up-regulation of the cell cycle inhibitor CDKN1A (16, 17). Interestingly, frequent allelic loss has been reported on the long arm of chromosome 16q22, where the \textit{ATBF1} gene is located, in hepatocellular carcinoma (18) and breast cancer (19). In addition, frequent inactivating mutations of the \textit{ATBF1} gene were reported in prostate cancer (20). Thus, we hypothesized that the \textit{ATBF1} gene is a candidate tumor suppressor gene in gastric cancer at 16q22 and that genetic alteration of the \textit{ATBF1} gene may contribute to the production of AFP in gastric cancers.

Therefore, to investigate whether somatic changes of the \textit{ATBF1} gene are involved in the development or progression of gastric cancers and the production of AFP in gastric cancer cells, we have analyzed AFP expression by immunohistochemistry; in addition, we studied genetic alterations, including mutations and loss of heterozygosity (LOH), of the \textit{ATBF1} gene in 81 sporadic gastric cancers.

\textbf{Materials and Methods}  
\textbf{Samples.} Eighty-one methacarn-fixed gastric cancer specimens were examined in this study. Six tumors were early gastric cancers, which were limited to the gastric mucosa and submucosa. Seventy-five tumors...
were advanced gastric cancers that invaded through the muscularis propria into the serosa. Histologically, 45 cases were the intestinal type and 36 were the diffuse type of gastric cancer. Informed consent was obtained from all the patients whose tumors were used in the study, according to the Declaration of Helsinki. Approval was obtained from the institutional review board of The Catholic University of Korea, College of Medicine. There was no evidence of familial cancer in any of the patients.

**Immunohistochemistry for the AFP.** For the immunohistochemical analysis, 2-μm sections were cut the day before use and stained according to standard protocols. To maximize the signal on immunohistochemistry, two strategies were used in the present study, antigen retrieval in citrate buffer and signal amplification with biotinylated tyramide. For the former, heat-induced epitope retrieval was conducted by immersing the slides in Coplin jars filled with 10 mmol/L citrate buffer (pH 6.0) and boiling the buffer for 30 min in a pressure cooker (Nordic Ware) inside of a microwave oven at 700 W; the jars were then cooled for 20 min. For the latter, the Renaissance TSA indirect kit (NEN Life Science), which included streptavidin-peroxidase and biotinylated tyramide, was used. After rinsing with PBS, the slides were treated with 1% H2O2 in PBS for 15 min at room temperature to abolish endogenous peroxidase activity. After washing with TNT buffer [0.1 mol/L Tris-HCl (pH 7.4), 0.15 mol/L NaCl, 0.05% Tween 20] for 20 min, the slides were treated with TNB buffer [0.1 mol/L Tris-HCl (pH 7.4), 0.15 mol/L NaCl, 0.5% blocking reagent]. Sections were incubated overnight at 4°C with the antibody (1:100 dilution) for AFP protein (DakoCytomation). Detection was carried out using biotinylated goat anti-rabbit antibody (Sigma) followed by incubation with peroxidase-linked avidin-biotin complex. Diaminobenzidine was used as a chromogen, and the slides were counterstained with Mayer’s hematoxylin. The specificity of anti-AFP antibody was confirmed in nine cancer cell lines by Western blot analysis (data not shown). Staining for the AFP antigen was determined as positive when >5% of the cytoplasm stained positively. Two pathologists reviewed the results independently. As negative controls, the slides were treated with replacement of the primary antibody by nonimmune serum.

**Microdissection and DNA extraction.** The tumor cells were selectively procured from H&E-stained slides using a laser microdissection device (ION LMD, JungWoo International Co.). The surrounding normal gastric mucosal cells were also obtained to study the corresponding normal DNA from the same slides in all cases. DNA extraction was done using a modified single-step DNA extraction method as described previously (21).

**Single-strand conformation polymorphism and DNA sequencing.** Because most of the ATBF1 mutations in prostate cancer were detected in exons 8, 9, and 10 of the gene, genomic DNAs from each of the cancer cells as well as corresponding noncancerous gastric mucosal tissues were amplified with primers covering the glutamine/proline–rich domain of the gene as described previously (20). Numbering of the sequences of ATBF1 was done with respect to the ATG start codon according to the genomic sequence obtained from Genbank accession no. NM_006885. All cases were screened by single-strand conformational polymorphism (SSCP) analysis of each exon for the presence of an aberrant band in tumor DNA compared with the normal DNA. Each PCR procedure was done under standard conditions in a 10 μL reaction mixture containing 1 μL of the template DNA, 0.5 μmol/L of each primer, 0.2 μmol/L of each deoxynucleotide triphosphate, 1.5 mmol/L MgCl2, 0.4 unit AmpliTaq gold polymerase (Perkin-Elmer), 0.5 μCi [3H]dCTP (Amersham), and 1 μL of 10× buffer. The reaction mixture was denatured at 94°C for 12 min and then incubated for 35 cycles (denaturing at 94°C for 40 s, annealing at 52-62°C for 40 s, and extension at 72°C for 40 s). A final extension step at 72°C was done for 5 min. After amplification, the PCR products were denatured for 5 min at 95°C in a 1:1 dilution of sample buffer containing 90% formamide/5 mmol/L NaOH. These products were loaded onto a SSCP gel (FMC Mutation Detection Enhancement System, Intermountain Scientific) containing 10% glycerol. After electrophoresis, the gels were transferred to 3 MM Whatman paper and dried. Autoradiography was then done using Kodak X-OMAT film (Eastman Kodak). The DNAs showing mobility shifts were cut out from the dried gels and amplified for 35 cycles using the same primer set. Sequencing of the PCR products was carried out using the cycle sequencing kit (Perkin-Elmer) according to the manufacturer’s recommendation.

**LOH analysis for the ATBF1 gene locus.** This study used two microsatellite markers, D16S3139 and D16S3066, which were localized...
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to within −0.25 and −0.11 Mb of the ATBF1 gene locus, respectively (20). The tumor and the corresponding noncancerous gastric mucosa DNAs were amplified using a thermal cycler (MJ Research Institute) with the two microsatellite markers. Each PCR was done under standard conditions in a 10 µL reaction mixture containing 20 ng of the template DNA, 0.5 µM of each primer, 0.2 µM of each deoxynucleotide triphosphate, 1.5 mmol/L MgCl₂, 0.4 unit Taq polymerase, 0.5 µCi [³²P]dCTP, and 1 µL of 10× buffer. The PCR products were then denatured and electrophoresed in a 6% polyacrylamide gel containing 7 mol/L urea. After electrophoresis, the gel was transferred to 3 MM Whatman paper, dried, and subjected to autoradiography using a Kodak X-OMAT film. The samples showing allelic shift at these markers in the tumor, compared with adjacent normal tissue, were scored as microsatellite instability; the complete absence of one allele in the tumor DNA, of the informative cases as defined by direct visualization, was considered to represent LOH.

Statistical analysis. The χ² test for association was used to test the relationship between genetic alterations of the ATBF1 gene and AFP expression in gastric cancer. A P value <0.05 was considered statistically significant.

Results

Immunohistochemistry of AFP expression. Moderate to strong immunopositivity for AFP was clearly marked on the cytoplasm and focal membrane of gastric cancer cells (Fig. 1). However, corresponding gastric mucosal epithelial cells and surrounding stromal cells including fibroblasts were negative for AFP. Expression of AFP protein was detected in 8 (9.9%) of the 81 gastric cancers, all were advanced gastric cancers. Histologically, five were the intestinal-type gastric cancer and three were the diffuse-type gastric cancer. Six AFP-producing cancers were cases with lymph node metastasis (Table 1) and three metastatic cancer cells in lymph nodes were positive for AFP (Fig. 1).

Mutation analysis of the ATBF1 gene. Enrichment and sequencing analysis of aberrant migrating bands on the SSCP gel identified ATBF1 mutations in seven (8.6%) of the samples (Fig. 2A-D). As shown in Fig. 2 and Table 1, one of the mutations was a missense mutation caused by a single nucleotide substitution, A to G transition at nucleotide 8387 (Fig. 2). As expected, two cases with a mutation and allelic loss at the ATBF1 locus, 3 cases showed allelic loss at these markers (Fig. 2E). Two of the cases with the ATBF1 mutation showed allelic loss at these markers, which was consistent with the SSCP data. Of 27 cases with allelic loss at the ATBF1 locus, 3 cases showed AFP protein expression in cancer cells. Statistically, there was no significant correlation between AFP expression and allelic loss at the ATBF1 locus (P = 0.9495, χ² test). As expected, two cases with a mutation and allelic loss in the ATBF1 gene showed AFP expression (Table 1).

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Sex/age</th>
<th>Histology</th>
<th>Differentiation</th>
<th>Lymph node metastasis</th>
<th>TNM Size (cm)</th>
<th>Site</th>
<th>AFP</th>
<th>Allelic loss</th>
<th>Mutation</th>
<th>Predicted effect</th>
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<td>21</td>
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<td>4.5</td>
<td>B</td>
<td>+</td>
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<tr>
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<td>MD</td>
<td>II9S</td>
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<td>+</td>
<td>-</td>
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<td>II</td>
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<tr>
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<td>Q1740del</td>
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</table>

Abbreviations: Diff., differentiation; MD, moderate differentiation; PD, poor differentiation; WD, well differentiation; TNM, tumor-node-metastasis classification; A, antrum; B, body; C, cardia; NI, noninformative.

1Retention of heterozygosity.
2Loss of heterozygosity.
AFP is frequently detected in hepatocellular carcinoma and yolk sac tumors (3). Gastric cancers also produce AFP in cancer cells, normally suppressed after birth. AFP production has been associated with tumor lymphatic and venous invasion and liver metastasis. Thus, AFP production in cancer cells seems to be an important marker for a poor prognosis (3). In the present study, the molecular mechanisms involved in AFP expression in gastric cancer cells were investigated. We examined AFP expression in gastric cancer tissue and genetic alteration of the ATBF1 gene, which has been characterized as a tumor suppressor gene and a negative regulator of the AFP gene. The immunohistochemical studies showed that AFP expression was present in eight cases with advanced gastric cancer tissues and six of them had lymph node metastasis. These findings further support AFP-producing gastric cancer as highly metastatic compared with non-AFP-producing gastric cancer.

Several studies have presented evidence that the ATBF1 gene down-regulates AFP and Myb (15, 16) and transactivates the cell cycle inhibitor CDKN1A (22, 23). In addition, it has been reported that the AFP expression in gastric cancer is due to the lack of the transcription factor ATBF1 (24), not to methylation of the AFP promoter region. Recently, frequent inactivating mutations of the gene were found in human prostate cancer; germ-line mutations were significantly associated with prostate cancer risk among sporadic cases (20, 25). Therefore, it is reasonable to consider that inactivation of the ATBF1 gene, through mutation or reduced expression, may be involved in gastric carcinogenesis, not only as a tumor suppressor gene but also by allowing gastric cancer cells to produce AFP protein. Thus, we examined the somatic changes of the ATBF1 gene in gastric cancer tissues.

ATBF1 mutations were found in 7 (8.6%) of 81 gastric cancers. There was one case with a missense mutation and six cases with deletion mutations and amino acid loss (Table 1). Four of seven gastric cancers with an ATBF1 mutation were advanced cancers with lymph node metastasis. In particular, six of the mutations were detected in a glutamine/proline-rich domain of the gene, similar to prostate cancers (20). Interestingly, the ATBF1 mutations occurred frequently in gastric cancers with AFP expression (P = 0.0004). Because ATBF1 inhibits cell proliferation and such domains are common transactivation domains (20, 26), it is possible that the ATBF1 mutations detected in this study may contribute to the development or progression of gastric cancer and that inactivating mutations of ATBF1 may be involved in AFP expression in gastric cancer cells. Our results may underestimate the prevalence of ATBF1 somatic mutations in gastric cancer, as we searched for mutations only in the hotspot exons (8–10). Thus, further studies are necessary to analyze the genetic alterations in other regions, such as other coding exons, the promoter and splice sites, as well as promoter methylation.

For the LOH analysis, allelic loss at the ATBF1 locus was found in 27 (52.9%) of 51 informative cases at one or both markers. Two cases with allelic loss carried a somatic mutation of the ATBF1 gene, suggesting biallelic inactivation. Five cases with mutation and/or allelic loss showed AFP expression in cancer cells; the other cases did not express AFP. In addition, the cases with inactivation of both ATBF1 alleles produced AFP in cancer cells. There was no ATBF1 genetic alteration in three AFP-producing gastric cancers. We did not analyze the methylation status of the AFP and ATBF1 promoter region because of the small amount of DNA from the microdissected cells. However, it is likely that the AFP expression in the gastric cancer cells depends partially on the methylation status of the AFP promoter and partially on the negative regulator, ATBF1 gene, as described previously (24, 27, 28). In addition, there are several additional possibilities to explain AFP expression in gastric cancer cells: (a) mutations in other exons of the ATBF1 gene may lead to AFP expression in gastric cancers; (b) DNA hypermethylation of the ATBF1 gene (24); and (c) the ATBF1 gene may yield two variant mRNAs by alternative splicing and the use of independent promoters. In the regulation of AFP expression in hepatoma cell lines, the NH2-terminal region of ATBF1-A molecule is closely associated with the transcriptional repressor activity and ATBF1-B functions in a dominant-negative manner against ATBF1-A (18). (d) It is also possible that the remaining wild-type ATBF1 allele down-regulates AFP expression in cases with ATBF1 mutations or allelic loss. Thus,
further analysis of ATBF1 gene regulation in gastric cancer is needed.

Even with the small number of cases studied here, we conclude that genetic alterations in the ATBF1 gene play an important role in gastric cancer carcinogenesis and AFP expression. Additional studies with a larger patient cohort are needed to verify these initial observations. Functional analysis will certainly broaden our understanding not only of the pathogenesis of gastric cancer but also of the mechanism involved in AFP production in cancer cells.

References
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