Expression and subcellular localization of AT motif binding factor 1 in colon tumours

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Abstract. AT motif binding factor 1 (ATBF1) is a transcriptional regulator that functions as a tumour suppressor to negatively affect cancer cell growth. In the present study four specific polyclonal antibodies against ATBF1 were generated, and the expression and intracellular localization of ATBF1 in colon tumours and normal mucosa were investigated. The four polyclonal antibodies produced were as follows: MB34 and MB49, which recognize the N- and C-terminal fragments of ATBF1, respectively; and DI-120 and MB44, which recognize the middle fragments of ATBF1 that contain three nuclear localization signals (NLS). In total, 191 colon samples were examined by immunohistochemical analysis. In addition, colon cancer cells were transfected with four ATBF1 expression vectors, and the subcellular localization of each fragment was examined. Normal colon mucosal cells were not observed to express ATBF1. However, a small number of hyperplastic polyps, serrated adenomas and adenocarcinoma tissues were observed to express D1-120- and MB44-reactive middle segments of ATBF1. Colon cancer cells were observed to express DI-120- and MB44-reactive middle fragments of ATBF1 in their cell nuclei. However, the N- and C-terminal fragments of ATBF1 did not translocate to the nucleus. Transfection of ATBF1 fragments revealed cleavage of the ATBF1 protein and nuclear translocation of the cleaved middle portion containing the NLS. A positive correlation between the cytoplasmic localization of the N- and C-termini of ATBF1, nuclear localization of the middle portion of ATBF1 and malignant cancer cell invasion was observed. In conclusion, the results of the present study suggest that alterations in the expression and subcellular localization of ATBF1, as a result of post-transcriptional modifications, are associated with malignant features of colon tumours.

Introduction

AT motif binding factor 1 (ATBF1) was originally identified as an inhibitory transcription regulator of the α-fetoprotein (AFP) gene (1–4). ATBF1 competes with hepatocyte nuclear factor 1 (HNF1) to bind to AT-rich elements in the enhancer and promoter regions of the AFP gene. There are two isoforms of ATBF1, ATBF1-A and ATBF1-B, which are produced by alternative splicing (4). ATBF1-A is a 404-kDa protein containing four homeodomains, 23 zinc finger motifs and a number of segments thought to be involved in transcriptional regulation. ATBF1-B is a 306-kDa protein that possesses the same four homeodomains, however, it contains five fewer zinc finger motifs due to the absence of 920 amino acid residues at the N-terminus. ATBF1-B binds to AT-rich enhancer elements in the region flanking the promoter of the AFP gene and downregulates promoter activity. ATBF1 negatively regulates cancer cell growth (5), and a number of genetic alterations to ATBF1 have been reported in several cancers (6). ATBF1 is currently recognized as a novel tumour suppressor (7).

Due to the role of ATBF1 in transcriptional regulation, it is required to translocate from the cytoplasm to the nucleus. In a previous study investigating the subcellular localization of ATBF1 in gastric cancer, ATBF1 was demonstrated to bind to the AT motif in the promoter region of the mucin 5AC gene and negatively regulate its transcription (8). In addition, ATBF1 was observed to translocate to the nucleus by forming a complex with runt domain transcription factor 3 (RUNX3), in response to transforming growth factor (TGF)-β signal transduction (9). Previous studies have demonstrated that the subcellular localization of ATBF1 may be a potential prognostic maker for skin cancer and head and neck cancer (10,11). However, information regarding the post-transcriptional modifications of the ATBF1 protein and their association with the nuclear translocation of ATBF1 remain to be elucidated.

Key words: AT motif binding factor 1, colon cancer, nuclear localization signals, transcription factor

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In order to investigate the subcellular localization of ATBF1 and its post-transcriptional modifications in detail, four different polyclonal antibodies raised against four individual epitopes of the ATBF1 protein were generated. These were used for the immunohistochemical analysis of different types of colon cancer tissue samples, in order to determine the subcellular localization and post-transcriptional modifications of ATBF1 in colon cancer cells.

Materials and methods

Polyclonal antibodies. As shown in Fig. 1, the following 4 anti-ATBF1-A rabbit polyclonal antibodies were produced against independent epitopes: MB34, which recognizes the N-terminal region of ATBF1 (amino acids, 238-255); DI-120, which recognizes a middle region of ATBF1 (amino acids, 2114-2147); MB44, which recognizes a middle region of ATBF1 (amino acids, 2229-2245); and MB49, which recognizes the C-terminal region (amino acids, 3410-3426). The antibodies were produced as described previously (12). The specificity of all the antibodies used for the purposes of this study was confirmed by western blot analysis in a previous study (12), using whole cell protein and fractionated protein lysates from the nucleus and cytoplasm.

Tissue samples. Immunohistochemical analysis was performed on 191 human colon samples obtained from endoscopic polypectomy, mucosal resection, submucosal dissection or surgical procedures from 111 patients admitted to Nagoya City University Hospital (Nagoya, Japan) from November 2006 to December 2010. The histological profiles of these samples are presented in Table I. The present study was performed in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Nagoya City University Graduate School of Medical Sciences (Nagoya, Japan; reference no. 00-00-1312). Written informed consent was provided by all patients prior to surgical procedures, endoscopic examinations or surgeries, and included an opt-out system.

Immunohistochemistry. Samples were fixed in 10% formalin for immunohistochemical examination using the four aforementioned polyclonal antibodies raised against ATBF1-A. All the following incubations were performed at room temperature. Consecutive tissue sections (4-µm in thickness) were deparaffinized in xylene and hydrated using a graded ethanol series. Antigens were retrieved in a pressure cooker with citrate buffer (0.01 M, pH 6.0) at 110 °C for 4 min, sections were subsequently cooled down to room temperature. The sections were incubated with methanol containing 0.3% H2O2 and 1.0% sodium azide for 5 min to block endogenous peroxidase activity. Antibodies were diluted with Ready-to-Use Dako Antibody Diluent (S0809; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) containing carrier protein to reduce background binding as follows: MB34, 1:1,500; DI-120, 1:3,000; MB44, 1:1,200; MB49, 1:500. The sections were incubated with the primary antibodies for 60 min, washed in PBS and covered with undiluted EnVision+ System HRP Labelled Polymer (K4003; Dako; Agilent Technologies, Inc.) for 60 min. The immune complexes were visualized by incubating the samples with 0.01% H2O2 and 0.05% 3,3'-diaminobenzidine tetrachloride (DAB) for 4 min. Nuclei were counterstained with Mayer's haematoxylin for 20 sec. The level of background staining by was observed to be negligible in the absence of antibodies (data not shown). Slides were analyzed with a light microscope (Olympus BX53; Olympus Corporation, Tokyo, Japan). Immunostaining of the nuclei and the cytoplasm in target tissues (in a 250x250-µm area) with 20x objective lens was evaluated in samples selected at random, and analysed in triplicate.

A pathologist (S.S.) assessed the staining level by observation of the sections under a light microscope. The following scoring was employed: Negative staining=0; weak positive staining=1; strong positive staining=2. The difference between 1 and 2 was arbitrarily by the pathologist.

Immunofluorescence and confocal microscopic examination of the subcellular localization of ATBF1-A. The human colon cancer cell line HCT116 (ref. no. CCL-274; American Type Culture Collection, Manassas, VA, USA) was cultured in McCoy's 5A medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum and 1% ampicillin and streptomycin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cell line authentication by profiling of short tandem repeats was performed by

Table I. Histological profiles of the samples examined.

| Colon sample                  | No. of samples |
|-------------------------------|----------------|
| Normal colon mucosa           | 80             |
| Hyperplastic polyp            | 4              |
| Serrated adenoma              | 11             |
| Tubular adenoma               | 13             |
| Tubulovillous adenoma         | 17             |
| Tubular adenocarcinoma (tub 1)|                |
| m                             | 11             |
| sm                            | 22             |
| mp                            | 1              |
| ss                            | 4              |
| Tubular adenocarcinoma (tub 2)|                |
| m                             | 2              |
| sm                            | 4              |
| mp                            | 3              |
| ss                            | 10             |
| Poorly differentiated adenocarcinoma |          |
| mp                            | 1              |
| ss                            | 2              |
| Mucinous adenocarcinoma       |                |
| sm                            | 1              |
| ss                            | 5              |
| Total                         | 191            |

Legend: tub 1, well-differentiated adenocarcinoma; tub 2, moderately differentiated adenocarcinoma; m, mucosa; sm, submucosa; mp, muscularis propria; ss, subserosa.
The rate of positive nuclear and cytoplasmic staining for each of the four antibodies in tub1 and tub2 colon cancers is shown in Fig. 2. Expression of the middle region of ATBF1 fragment (detected using D1-120) was significantly correlated with invasion to the deep layer of the colon wall (D1-120 in tub2 nucleus, P=0.004) (Table III).

The next aim of the present study was to examine the association between ATBF1 expression (and/or localization) and cancer invasion in tub1 and tub2 cancer tissues. As indicated in Table III, N-terminal (MB34) and C-terminal (MB49) fragments of ATBF1 were highly expressed in the cytoplasm. The expression of N-terminal and C-terminal fragments were statistically significantly correlated with the cancer invasion depth (MB34 in tub1: P=0.004; MB49 in tub1: P=0.006). However, nuclear expression of N-terminal and C-terminal fragments was not detected. Furthermore, nuclear expression of the middle region of the ATBF1 fragment (detected using D1-120) was significantly correlated with invasion to the deep layer of the colon wall (D1-120 in tub2 nucleus, P=0.004) (Table III).

Results

ATBF1 expression in the colonic mucosa. As indicated in Table II, no ATBF1 expression was observed in normal colonic mucosa tissues using all four of the anti-ATBF1 antibodies. ATBF1 expression was almost completely absent from hyperplastic polyps and serrated adenomas, with positive expression detected in the nuclei of 25% hyperplastic polyps and 18% serrated adenomas using the MB44 antibody (Table II). Tubular adenomas were not observed to express ATBF1, however, a number of tubulovillous adenomas stained positive for the middle portion of ATBF1 (using D1-120 and MB44 antibodies) in the cytoplasm and nucleus (Table II). In particular, 41% of nuclei in tubulovillous adenomas were positive for MB44 staining. The expression of ATBF1 in the nucleus and cytoplasm, as detected using the D1-120 and MB44 antibodies, was significantly higher in adenocarcinoma tissues when compared with benign polyps and adenomas (P<0.001 for D1-120 and MB44 antibodies; Table II). Notably, all poorly differentiated adenocarcinomas were observed to express ATBF1 using the MB34, MB44 and MB49 antibodies, with 100% nuclear expression detected using the MB44 antibody (Table II). Comparisons between tub 1 and tub 2 tubular adenocarcinomas revealed significantly higher ATBF1 expression in tub 2 compared with tub 1 adenocarcinomas using the D1-120 and MB44 antibodies (P=0.01; Table II).

Correlation between ATBF1 expression and colon cancer invasion. The expression of the middle region of the ATBF1 fragment (detected using D1-120) was significantly correlated with invasion to the deep layer of the colon wall (D1-120 in tub2 nucleus, P=0.004) (Table III).

The rate of positive nuclear and cytoplasmic staining for each of the four antibodies in tub1 and tub2 colon cancers is shown in Fig. 2. Expression of the middle region of ATBF1 was increased in the cytoplasm and nucleus with increasing depth of invasion, whereas, increases in the expression of the N- and C-terminal regions of ATBF1 were observed in the cytoplasm (Fig. 2). The depth of invasion was assigned the following values: Mucosa, 0; submucosa, 1; muscularis propria, 2; subserosa, 3. Univariate regression analysis revealed significant correlations between the depth of tumour invasion and cytoplasmic ATBF1 expression detected using MB34 (P=0.002), D1-120 (P=0.001), MB44 (P=0.022) and MB49 (P=0.002) antibodies. Furthermore, significant correlations were observed between the depth of tumour invasion and nuclear expression when using D1-120 (P<0.001) and MB44
Table II. Rate of positive staining for ATBF1 fragments in different colon samples using four types of anti-ATBF1 polyclonal antibody.

| Colon tissue                        | Normal mucosa (n=80) | Hyperplastic polyp (n=4) | Serrated adenoma (n=11) | Tubular adenoma (n=13) | Tubulovillous adenoma (n=17) | Tubular adenocarcinoma tub1 (n=38) | Tubular adenocarcinoma tub2 (n=19) | Poorly differentiated adenocarcinoma (n=3) | Mucinous adenocarcinoma (n=6) |
|-------------------------------------|----------------------|--------------------------|-------------------------|------------------------|------------------------------|-----------------------------------|-----------------------------------|---------------------------------------|--------------------------|
| Anti-ATBF1 antibody                 |                      |                          |                         |                        |                              |                                    |                                   |                                        |                          |
|                                    | MB34 (%)             | D1-120 (%)               | MB44 (%)                | MB49 (%)               |                              |                                    |                                   |                                        |                          |
|                                     | Nucleus               | Cytoplasm                | Nucleus                 | Cytoplasm              | Nucleus                      | Cytoplasm                         | Nucleus                           | Cytoplasm                             | Nucleus                  |
| Normal mucosa (n=80)                | 0                     | 0                        | 0                       | 0                      | 0                            | 0                                  | 0                                 | 0                                      | 0                        |
| Hyperplastic polyp (n=4)            | 0                     | 0                        | 0                       | 0                      | 0                            | 0                                  | 0                                 | 0                                      | 0                        |
| Serrated adenoma (n=11)             | 0                     | 0                        | 0                       | 0                      | 0                            | 0                                  | 0                                 | 0                                      | 0                        |
| Tubular adenoma (n=13)              | 0                     | 0                        | 0                       | 0                      | 0                            | 0                                  | 0                                 | 0                                      | 0                        |
| Tubulovillous adenoma (n=17)        | 0                     | 0                        | 0                       | 0                      | 0                            | 0                                  | 0                                 | 0                                      | 0                        |
| Tubular adenocarcinoma              |                      |                          |                         |                        |                              |                                    |                                   |                                        |                          |
| tub1 (n=38)                         |                      |                          |                         |                        |                              |                                    |                                   |                                        |                          |
|                                     | 0                     | 58                       | 29<sup>a</sup>          | 26<sup>a</sup>         | 53<sup>b</sup>              | 42<sup>b</sup>                    | 0                                 | 55                                      |                          |
| tub2 (n=19)                         | 0                     | 84                       | 63<sup>a</sup>          | 84<sup>a</sup>         | 74<sup>b</sup>              | 74<sup>b</sup>                    | 0                                 | 84                                      |                          |
| Poorly differentiated adenocarcinoma|                      |                          |                         |                        |                              |                                    |                                   |                                        |                          |
| (n=3)                               | 0                     | 100                      | 33<sup>a</sup>          | 67<sup>a</sup>         | 100<sup>b</sup>             | 100<sup>b</sup>                   | 0                                 | 100                                     |                          |
| Mucinous adenocarcinoma (n=6)       | 17                    | 50                       | 50<sup>b</sup>          | 50<sup>b</sup>         | 83<sup>b</sup>              | 33<sup>b</sup>                    | 17                                | 33                                      |                          |

MB34 recognizes the N-terminal region of ATBF1 (amino acids 238-255); D1-120 recognizes the middle region of ATBF1 (amino acids 2,114-2,147); MB44 recognizes the middle region of ATBF1 (amino acids 2,229-2,245); and MB49 recognizes the C-terminal region of ATBF1 (amino acids 3,410-3,426). ATBF1 expression was significantly higher in the nucleus and cytoplasm in tumour samples compared with benign polyps and adenomas (*P<0.001 and **P<0.001). ATBF1, AT motif binding factor 1; tub1, well-differentiated adenocarcinoma; tub2, moderately differentiated adenocarcinoma.
antibodies, but not when using MB34 (P=1.00) and MB49 (P=1.00) antibodies. Representative images of immunohistochemical staining of colon cancer specimens using the four anti-ATBF1 antibodies are shown in Fig. 3.

Table III. Rate of positive staining for ATBF1 fragments in tubular adenocarcinoma of the colon using four types of anti-ATBF1 polyclonal antibodies.

| Tubular adenocarcinoma | MB34 (%) | D1-120 (%) | MB44 (%) | MB49 (%) |
|------------------------|----------|------------|----------|----------|
|                        | Nucleus  | Cytoplasm  | Nucleus  | Cytoplasm | Nucleus  | Cytoplasm | Nucleus  | Cytoplasm |
| tub1                    |          |            |          |          |          |          |          |          |
| m (n=11)                | 0        | 27         | 36       | 18       | 46       | 27       | 0        | 27       |
| sm (n=22)               | 0        | 63         | 14       | 23       | 45       | 45       | 0        | 59       |
| mp (n=5)                | 0        | 100        | 80       | 60       | 100      | 60       | 0        | 100      |
| tub2                    |          |            |          |          |          |          |          |          |
| m (n=2)                 | 0        | 50         | 0        | 100      | 50       | 50       | 0        | 50       |
| sm (n=4)                | 0        | 50         | 25       | 50       | 25       | 50       | 0        | 50       |
| mp (n=13)               | 0        | 100        | 85       | 92       | 92       | 85       | 0        | 100      |

MB34 recognizes the N-terminal region of ATBF1 (amino acids 238-255); D1-120 recognizes the middle region of ATBF1 (amino acids 2,114-2,147); MB44 recognizes the middle region of ATBF1 (amino acids 2,229-2,245); and MB49 recognizes the C-terminal region of ATBF1 (amino acids 3,410-3,426). ATBF1 expression was significantly correlated with the depth of cancer invasion (P=0.004, P=0.004 and P=0.006). ATBFT, AT motif binding factor 1; tub1, well-differentiated adenocarcinoma; tub2, moderately differentiated adenocarcinoma; m, mucosa; sm, submucosa; mp, muscularis propria.

Figure 2. Level of positive staining for ATBF1 in well-differentiated and moderately differentiated tubular adenocarcinomas of the colon, as determined by immunohistochemical analysis using four anti-ATBF1 polyclonal antibodies. Positive correlations between the depth of tumour invasion and ATBF1 expression were observed, except for nuclear ATBF1 expression as determined using the MB34 and MB49 antibodies. ATBF1, AT motif binding factor 1; m, mucosa; sm, submucosa, mp, muscularis propria; ss~, subserosa.

(P=0.002) antibodies, but not when using MB34 (P=1.00) and MB49 (P=1.00) antibodies. Representative images of immunohistochemical staining of colon cancer specimens using the four anti-ATBF1 antibodies are shown in Fig. 3.

Putative role of the middle region of ATBF1 in nuclear translocation. In order to confirm the mechanism underlying ATBF1 nuclear translocation, the HCT116 colon cancer cells were transfected with the four HA-tagged ATBF1 expression vectors, and subcellular localization was evaluated using confocal microscopy (Fig. 4). As shown in Fig. 4A, the HA-ATBF1 (full-length ATBF1) and ATBF1-7.2M expression vectors encompass 3 nuclear localization signals (NLS), namely NLS1387, NLS2947 and NLS2987, whereas
the ATBF1-2.7N and ATBF1-1.2C expression vectors do not encompass any NLS. Confocal microscopy analysis revealed that HA-ATBF1 and ATBF1-7.2M translocated to the nucleus, whereas ATBF1-2.7N and ATBF1-1.2C were localized in the cytoplasm (Fig. 4B). Based on these observations, the authors speculate that the ATBF1 N- and C-terminal fragments detected by the MB34 and MB49 antibodies may have been cleaved post-transcriptionally, and were therefore unable to translocate to the nucleus as they lack NLS. By contrast, the full-length and middle regions of ATBF1, detected by the
D1-120 and MB44 antibodies, were able to translocate to the nucleus due to the presence of NLS.

Discussion

In the present study a positive association between ATBF1 expression and the step-wise process of carcinogenesis in the colonic mucosa was observed, and the level of ATBF1 expression increased with the extent of tumour invasion to the colonic wall. In addition, the N- and C-terminal regions of ATBF1 were demonstrated to not translocate to the nucleus. This suggests that the ATBF1 protein is cleaved and the N- and C-terminal fragments of ATBF1 are released from the remainder of ATBF1. These fragments remain in the cytoplasm as they lack NLS. A previous study reported that nuclear translocation of ATBF1 is associated with TGF-β signal transduction and/or RUNX3 (9). In addition, sumoylation is involved in the nuclear localization of ATBF1 (13). The authors of the present study are currently investigating the key enzyme that cleaves ATBF1, as an additional factor that may contribute to its subcellular localization.

As reported previously, ATBF1 is a novel tumour suppressor protein that interacts with p53 and Myb (14,15). Together with the findings of the present study, these observations indicate that increased ATBF1 expression may be associated with increased levels of p53 or Myb in colon cancer cells.

A number of ATBF1 mutations have been reported in prostate cancer (7), however, there is limited information regarding ATBF1 gene mutations in colon cancer. There are two potential reasons for the increased expression of ATBF1 in colon cancer during the process of carcinogenesis. Firstly, as ATBF1 is a tumour suppressor, its expression may increase in an attempt to inhibit cell cycle progression and suppress cancer cell growth (7,16). Secondly, it is possible that abnormal ATBF1 proteins, which lack the normal function of ATBF1, exhibit a dominant negative effect, which leads to cancer development (12). Future studies should therefore investigate genetic mutations of ATBF1 in colon cancer. The molecular mechanism of the regulation of ATBF1 gene expression has been reported previously (17). ATBF1 contains several DNA binding domains including four homeodomains and twenty-three zinc finger domains (4). In addition to these DNA binding domains ATBF1 has distinct protein-protein interaction domains to bind Myb (14), p53 (15) and protein inhibitor of activated STAT3 (PIAS3) (18). These protein-protein interactions suggest ATBF1 may function as a suppressive factor for tumor progression. Without these co-factors, ATBF1 does not function as a tumor-suppressor. For example, ATBF1 activates p21(Waf1/Cip1) to halt the cell cycle only when it interacts with p53 (15). The fragmentation of ATBF1 may interfere with the full length ATBF1 and prevent the effective suppression of malignancy in cancer cells. The lack of ATBF1 expression has been reported in the majority of malignancies (12) and embryonic carcinomas (16). Notably, improved clinical prognosis has been observed with the expression of ATBF1 fragments compared with no expression (12). However, it is not known whether the ATBF1 fragments are responsible, or whether unknown mechanisms besides ATBF1 serve an oncosprespresive role in these cases.

The authors of the present study hypothesize that fragments of ATBF1 may function as dominant negative factors for the same target genes. ATBF1 was initially identified as a suppressor of the AFP gene. A previous study demonstrated that a fragment of ATBF1 was unable to suppress AFP (19). The cytoplasmic expression of ATBF1 fragments in hepatocellular carcinomas may not suppress AFP. ATBF1 contains three nuclear localization signals (NLSs) (12). NLSs are distinct motifs from the DNA binding domains on the ATBF1 (4). The ATBF1 fragments localized in the cytoplasm that contain DNA binding domains, but not the NLSs, will have no chance to interact with DNA because the target DNA is exclusively localized in the nucleus. Nuclear localization of ATBF1 is an important factor in the improved prognosis of patients with bladder carcinomas (12). However, it is possible that ATBF1 fragments present in the cytoplasm interact with PIAS3 to inhibit the signal transduction of signal transducer and activator of transcription (STAT)3 (18); suppression of the STAT3 inflammatory reaction may limit the progression of malignant tumors. Janus kinase (JAK)-STAT3 signaling promotes cancer progression through inflammation, obesity, stem cells and the pre-metastatic niche (20). The JAKs and STAT proteins, particularly STAT3, are among the most promising new targets for cancer therapy (20).

In conclusion, the results of the present study demonstrate that ATBF1 expression increases during the malignant progression of colon cancer. In addition, the nuclear localization of ATBF1 is regulated by three NLS located in the middle region of ATBF1. The C- and N-terminal fragments of ATBF1, which lack NLS, remain in the cytoplasm of colon cancer cells. Cleavage of ATBF1 increases the level of cytoplasmic ATBF1 fragments. Alterations in the subcellular localization of ATBF1, due to its fragmentation, are associated with malignant features of colon cancer.

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