Potential Dual Role of Activating Transcription Factor 3 in Colorectal Cancer

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Abstract. Background/Aim: Activating transcription factor 3 (ATF3) is a member of the ATF/CREB transcription factor family and has been proposed as a molecular target for cancer therapy. The present study was undertaken in order to investigate whether ATF3 influences cancer-related phenotypes in colorectal cancer. Materials and Methods: ATF3 was overexpressed in human colorectal cancer cells and the effects of ATF3 on apoptosis, cell cycle, cell migration and epithelial mesenchymal transition (EMT) were investigated. B-cell lymphoma-2 (Bcl-2) promoter was cloned and used for luciferase assay in cells transfected with control or ATF3 expression vector. Results: ATF3 down-regulated the expression of Bcl-2 and promoter activity of the Bcl-2 gene. ATF3 increased collective cell migration and expression of cluster of differentiation 44 (CD44) and decreased retinoblastoma (Rb) expression. In addition, ATF3 down-regulated EMT-inducing transcription factors and β-catenin. Conclusion: ATF3 may play a dichotomous role in apoptosis and metastasis in human colorectal cancer cells.

Colorectal cancer (CRC) occupies the third place in cancer incidence and mortality in the United States (1). Understanding of molecular targets is very important for the development of effective prevention and therapeutic strategies. During carcinogenesis, cells undergo and respond to numerous cellular and physical stresses, and the failure to restrain and eliminate stress signals can increase the risk of cancer (2). Activating transcription factor 3 (ATF3) is a member of the ATF/CREB family and contains basic region-leucine zipper (bZip) DNA binding domain (3). Basal expression of ATF3 is low in normal cells and can be induced by various stress stimuli and signals that damage cells or tissues (4). These stress signals include hypoxia, anoxia, carcinogens, DNA damage, UV exposure and radiation (5). In addition, ATF3 is responsible for adaptation to different extra- and intra-cellular stimuli (6, 7).

In previous studies, we found that ATF3 could be a molecular target of many anticancer compounds such as PI3K inhibitor (8), epicatechin gallate (9), indole-3-carbinol (10), conjugated linoleic acid (11), and tolfenamic acid (12), that mediate compounds-stimulated apoptosis (9-11). However, the role of ATF3 in cancer seems to be complex and dichotomous. Recently Dr. Kitajima’s group reported that ATF3 mediated ER stress-induced sensitization of colon cancer cells to TRAIL-mediated apoptosis (13, 14) and regulated synergistic anticancer activity of a HDAC inhibitor and anti-DR5 antibody in human colon cancer cells (15). Stable or transient overexpression of ATF3 increased caspase activity and enhanced the etoposide- or camptothecin-induced apoptosis in HeLa cells (16). However, ATF3 inhibited apoptosis through activating the AKT pathway in PC12 cells (17). Recently, Yin et al. reported that ATF3 enhanced apoptosis in the untransformed MCF10A mammary epithelial cells, whereas protected the aggressive MCF10CA1a cells and enhanced cell motility (18). In addition, an in vivo study using a xenograft mouse model showed that ATF3 possessed either tumor suppressive or oncogenic activity (19-22). The role of ATF3 in apoptosis depends on the cell type, tissue context and stage of tumorigenesis.

The current study was undertaken in order to investigate the effect of ATF3 expression on tumorigenicity to better understand the role of ATF3 in human colon cancer progression.

Materials and Methods

Cell culture and antibodies. Human colorectal adenocarcinoma cells (HCT116, HCT15, Caco-2, LoVo, HT29 and SW480) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and grown in Dulbecco’s modified Eagle’s medium (DMEM/F12) supplemented with 10% fetal bovine serum (FBS).
Recombinant human epithelial growth factor (EGF), basic fibroblast growth factor (bFGF) and propidium iodide (PI) were purchased from Sigma (St. Louis, MO, USA). The antibodies for Bcl-2 and ATF3 were purchased from BD Biosciences (San Jose, CA, USA) and Santa Cruz (Santa Cruz, CA, USA), respectively. Antibodies for β-actin, Bak, Bax, cyclin D1, p21, p27, Rb, CD44, ZO-1, GSK3 β, and β-catenin were purchased from Cell Signaling (Beverly, MA, USA).

Cloning of Bcl-2 promoter and measurement of luciferase activity. Human Bcl-2 promoter region spanning from -1000 to +715 base pairs was amplified from human genomic DNA by PCR. PCR primers used are as follows: (F: 5'-CGATCTAAGTCAGAGTATGATGATGAGGAC-3', R: 5'-CCAGGCTGAGGTATTCTTG-3'). PCR products were cloned into pGL3-basic vector (Promega, Madison, WI, USA) by using In-Fusion cloning method (Clontech, Mountain View, CA, USA). The luciferase activity was measured using a dual luciferase assay kit (Promega) as we described previously (12). The promoter activity was expressed as ratio of Bcl-2 promoter activity/pGL3-basic activity.

Cloning of ATF3 expression vector and establishing ATF3-expressing stable cell line. Human ATF3 plasmid DNA was amplified with following primers (F: 5'-CGATCTAAGTAAGCTCAGCTAAGGAC-3', R: 5'-CCAGGCTGAGGTATTCTTG-3') and PCR products were inserted into pAcGFP-C1 by using In-Fusion cloning method from Clontech. To create a cell clone overexpressing human ATF3 gene, the pAcGFP-C1-ATF3 vectors were transfected into HCT15 cells using Lipofectamine 2000, and the cells were maintained with G418 for two weeks to eliminate the untransfected cells.

Transient transfection and overexpression of ATF3. Transient transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) or PolyJet (SignaGen Laboratories, Rockville, MD, USA), as we previously described (12). The pCG-ATF3 expression construct was described previously (12). pcDNA3.1 V5/His empty vector were used as control. The cells were plated in 6-well plates at the concentration of 4x10⁵ cells/well and grown overnight. The next day, plasmid mixtures containing 2.5 μg of control or ATF3 expression vector were transfected for 48 h.

Western blot and RT-PCR analysis. Western blot and RT-PCR were performed as described previously (12). For western blot, Chemiluminescence was visualized by ChemiDoc MP Imaging system (Bio-Rad, Hercules, CA, USA). For RT-PCR, total RNA was extracted and cDNA was synthesized using Verso cDNA kit (Thermo Scientific). PCR was carried out using ReadyMix Taq polymerase (Sigma). The sequences of PCR primers are indicated in Table I.

Apoptosis and cell-cycle analysis. Apoptosis and cell-cycle analysis were measured using flow cytometer, as we described previously (23). For apoptosis, Annexin V positive/PI positive and Annexin V positive/PI negative cell populations were determined as apoptotic cells from the total gated cells.

Tumorsphere forming assay. Stable cells overexpression control or ATF3 expression vector were plated onto 1% methylcellulose on poly–HEMA coated 6-well plates at the concentration of 2x10⁴/well and grown in DMEM supplemented with 10% FBS, 20 ng/mL of human recombinant bFGF and 20 ng/mL of human recombinant EGF. Suspension cultures were performed for 14 days and then microscope images were taken by phase-contrast microscope.

Statistical analysis. Statistical analysis was performed with the unpaired Student’s t-test. Data were expressed as mean±SD and differences were considered significant at p≤0.05.

**Results**

**ATF3 modulates the expression of Bcl-2 family members in human colorectal cancer cells.** It is known that basal expression of ATF3 is low in normal cells and its expression is stimulated by stress and signals that damage cells (3). To observe differences in basal expression of ATF3 among different human colorectal cancer cell lines, we performed western blot analysis to compare protein levels of endogenous ATF3 in multiple human colorectal cancer cell lines with different genetic backgrounds. As shown in Figure 1, a low level of ATF3 was detected in HCT116, HCT15, HT29 and SW480, whereas higher expression of ATF3 was observed in Caco-2 and LoVo cells. RTPCR results also showed a similar pattern; low ATF3 mRNA in HCT116 and SW480 cells and increased mRNA in LoVo and Caco-2 cells (data not shown). In a previous study, we observed that overexpression of ATF3 induced apoptosis over 2-fold in HCT116 cells (8). To elucidate a potential anti-apoptotic mechanism of ATF3 gene, we tested if ATF3 overexpression affects the expression of Bcl-2 family proteins. Overexpression of ATF3 decreased the protein levels of Bcl-2 family members, including Bak, Bax, cyclin D1, p21, p27, Rb, CD44, ZO-1, GSK3 β, and β-catenin (Table II).
argin G1/S arrest in HeLa cells (24), we tested if ATF3 knockdown on cell cycle. Inversely, suppression of ATF3 in HCT116 cells resulted in minimal G1 arrest, and decreased minimal effect on the cell-cycle regulation.

(Figure 4B). These data suggest that ATF3 may show no or significant decrease of Bcl-2 and Bcl-2 band was not detected in SW480 cells (Figure 2A). RT-PCR data showed that Bcl-2 mRNA was decreased in HCT116, HCT15 and SW480 cells overexpressed ATF3 (Figure 2B). We also tested if knockdown of ATF3 reverses expression of Bcl-2. Knockdown of ATF3 using small interfering RNA (siRNA) elevated expression of Bcl-2 (data not shown). On the other hand, ATF3 overexpression increased the expression of proapoptotic protein, Bak in both protein and mRNA levels (Figure 2C and D). No changes were found in the expression of other Bcl-2 family members including Bax and Bcl-xL. Next, in order to investigate if decreased expression of Bcl-2 is associated with transcriptional down-regulation of the Bcl-2 gene, we cloned Bcl-2 promoter spanning from −1000 to +715 (Figure 3A). The promoter was co-transfected into HCT116 cells with control or ATF3 expression vector and then luciferase activity was measured. Results indicated a significant decrease of Bcl-2 promoter activity. Taken together, these data demonstrate that a decrease of Bcl-2 and an increase of Bak expression could be potential mechanisms of increased apoptosis in ATF3-overexpressing human colorectal cancer cells.

ATF3 does not affect cell cycle of human colorectal cancer cells. Since anticancer activity of ATF3 is mediated via increased G1/S arrest in HeLa cells (24), we tested if ATF3 overexpression changes sub-population of each cycle in human colorectal cancer cells. The fraction of each phase in cell cycle was analyzed using PI staining and FACS analysis in HCT116 cells transfected with control or ATF3 expression vector for 48 h. As shown in Figure 4A, ATF3 overexpression (HCT116 OA) resulted in a slight decrease in G1 phase and an increase in S and G2/M phase compared to control (HCT116 OC). We also tested the effect of ATF3 knockdown on cell cycle. Inversely, suppression of ATF3 in HCT116 cells resulted in minimal G1 arrest, and decreased S and G2/M phase. However, we did not observe any changes in cell cycle-regulatory genes such as cyclin D1 and cyclin-dependent kinase (CDK) inhibitors, p21 and p27 (Figure 4B). These data suggest that ATF3 may show no or minimal effect on the cell-cycle regulation.

ATF3 overexpression leads to an increase of collective cell invasion phenotype. During metastasis, invasive cancer cells divide rapidly, increase the size of primary tumor mass, invade the surrounding microenvironment and migrate. Cell migration occurs in two major modes. Single-cell migration with no cell-cell adhesion and collective cell migration with retained cell-cell junctions (25). To test the effect of ATF3 on the initial stage of metastasis, we established a stable cell line overexpressing ATF3 and analyzed the single cell migration using a Boyden chamber. The result indicates that ectopic expression of ATF3 in HCT116 and HCT15 cells does not affect single-cell motility (data not shown). Next, to test the effect of ATF3 on tumorigenic and metastasis potential of HCT116, we performed a tumor sphere-forming assay under non-adherent conditions. As shown in Figure 5A, ATF3-overexpressing HCT116 cells were typically approximately 200-300 μm in diameter after 14 days, whereas control HCT116 cells were 100-150 μm in diameter after 14 days. In addition, ATF3-overexpressing HCT116 cells showed more condensed cell density and collective invasion budding phenotype sites. Our result suggests that overexpression of ATF3 can promote tumor growth and collective invasion of human colorectal cancer cells. Tumorigenic cells are enriched in the fraction of cells that express high levels of CD44 (26). CD44 variant isoforms switching is associated to primary tumor growth and metastatic phenotype in colorectal cancer (27, 28). Thus, we performed western blotting to analyze the protein expression of CD44 and gain a better understanding over the role of ATF3 in colorectal cancer. As shown in Figure 5B, overexpression of ATF3 increased the expression of both standard and variant splice forms of CD44 and suppressed the expression of Rb and ZO-1 compared to control, respectively. These changes are crucial for initiating collective cell invasion and metastatic progression (29). CD44 splice variant switching is required to induce EMT in human epithelium (30).

Down-regulation of EMT-related genes by ATF3 in human colorectal cancer cells. EMT is an important cellular and molecular event during cancer progression and metastasis. We next examined whether ectopic expression of ATF3 in human colorectal cancer cells (HCT116, SW480 and HT29) affect the Snail family members Snail and Slug which has been known to trigger EMT during tumorigenesis (31). Unexpectedly, we found the Snail and Slug transcription factors were dramatically suppressed in HCT116, SW480 and HT29 cells transfected with ATF3 expression vector. The
activation of GSK3β can negatively regulate Snail and Slug (32, 33) and inhibit β-catenin expression (34). To determine the suppression of Snail and Slug transcription factors through regulation of GSK3β in ATF3-overexpressing HCT116, we examined the expression of GSK3β and β-catenin, another downstream target of GSK3β by western blot. As a result, ATF3 overexpression increased GSK3β and decreased β-catenin expression in HCT116 cells (Figure 6B). Our data suggested that ectopic expression of ATF3 promotes tumorsphere formation with collective invasion phenotype, but not EMT.

**Discussion**

ATF3 is a stress-inducible gene and responds to a variety of signals including DNA damage, hypoxia, anoxia, chemicals and microenvironment (3). Therefore, ATF3 is considered an adaptive-response gene that participates in cellular processes to adapt to extra- and/or intra-cellular changes and responds to signals disrupting homeostasis.

In this study, we cloned Bcl-2 promoter and tested if ATF3 directly influences Bcl-2 promoter activity. ATF3 suppressed the promoter activity and decreased the expression of Bcl-2 protein. Therefore, ATF3 may regulate Bcl-2 transcription by direct binding to the promoter region of Bcl-2. We identified three potential ATF3 binding sites in the promoter region we cloned (-841 to -830, -797 to -791, and -614 to -608). Further study using internal deletion clones of each CREB binding site is required to elucidate which cis-acting element is pivotal for ATF3-mediated Bcl-2 down-regulation. On the other hand, Bak, the pro-apoptosis protein from Bcl-2 family, is activated by ATF3.
Our data indicate that increase of ATF3 expression has a minimal effect on cell-cycle regulation and no changes in cell cycle-regulating proteins such as cyclins and cyclin-dependent kinases (CDKs) and CDK inhibitors, p21 and p27. However, Fan et al., reported that overexpression of ATF3 using the tetracycline-inducible system moderately reduced progression of cells from G1 to S phase in HeLa cells, indicating that the ATF3 protein might be a candidate in the control of cell-cycle arrest (24). This might be due to the different cell type and different induction system.

One of interesting findings of the current study is that ectopic expression of ATF3 in colon cancer cells increases invasion phenotype, a hub of biological network to promote cancer progression and metastasis. According to our result, increased tumorsphere formation and CD44-positive cells were also found in ATF3-overexpressing breast cancer cells (35). Recently, Wu et al. reported that knockdown of ATF3 in LoVo and Caco-2 cells suppressed in vitro cancer cell migration and invasion as well as in vivo tumor growth and liver metastasis (36). We also found that ATF3 overexpression decreased Rb and stimulated CD44, markers of colon cancer stem cell. A sub-population of cancer stem cells and interaction with stromal signals are pivotal for metastatic colonization and tumor expansion. To elucidate
Figure 5. ATF3 increases collective cell invasion. (A) Phase contrast image of tumorsphere-forming assay. HCT116 cells overexpressing control or ATF3 expression vector were used to test the collective invasion potency. (B) Western blot was performed with the indicated antibodies.

Figure 6. ATF3 down-regulates EMT inducers and β-catenin. (A) Human colorectal cancer cells (HCT116, SW480, HT29) were transfected with control or ATF3 expression vector and RT-PCR was performed for indicated genes. (B) Western blot was performed to analyze the expression level of indicated proteins in HCT116 cells transfected with control or ATF3 expression vector.
the mechanism of cell migration, further study to examine cell surface proteases including MMP2 is required, because this is an early event of collective cell movement and associated with ECM remodeling.

Another interesting finding is that ATF3 suppresses the expression of EMT-associated genes in human colorectal cancer cells. We speculate that enhanced tumorsphere-forming and invasion phenotype by ATF3 overexpression might be of an EMT-independent manner. Recently, EMT-independent migration pathways have been documented (37, 38). Herein, we propose two mechanisms. Firstly, ATF3 overexpression leads to the suppression of Wnt pathway with increase of GSK-3β and subsequently decrease of β-catenin expression (Figure 6B). In fact, GSK3β promotes proteasomal degradation of β-catenin and inhibits translocation of β-catenin to the nucleus and transcription of their target genes including EMT-suppressing genes (39). Secondly, ATF3 may suppress TGFβ-induced EMT. To test this hypothesis, we studied the effect of ATF3 overexpression on TGFβ-induced expression of EMT-related gene. However, we did not see morphological changes for EMT in human colorectal cancer cells treated with TGFβ (data not shown). It is probably due to mutation of TGFβ type II receptor in colorectal cancer (40).

Regarding the clinicopathological features of patients, Wu et al., observed that expression of ATF3 was much higher in colon tissues of cancer patients compared to matched non-cancerous colon tissues, supporting a potential cancer-promoting activity of ATF3 (36).

Interestingly, we observed that another colorectal cancer cell line, HCT15 showed different expression profile in EMT-related genes. Unlike HCT116, SW480 and HT29 cells, HCT15 cells had elevated Slug, Snail and vimentin mRNA expression level after ATF3 overexpression (data not shown). We do not know why HCT15 responds to ATF3 in a different way in terms of EMT. Further investigation is required to address this issue. Given the importance of ATF3 in in vitro cancer phenotypes, it is crucial to investigate how ATF3 functions in in vivo situation because mechanisms for biological activities of ATF3 are complex and different from in vitro conditions.

Conclusion

ATF3 inversely modulated the expression of Bcl-2 in different colon cancer cells. In addition, ectopic expression of ATF3 enhanced the tumorsphere-forming ability with collective invasion phenotype through activation of a variant from of CD44 and inactivation of Rb and ZO-1 protein and silencing of the EMT-associated transcription factors including Snail and Slug. Hence, these findings provide evidence to support a dual role of ATF3 in colorectal cancer progression.

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References
