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Atonal homolog 1 protein stabilized by tumor necrosis factor α induces high malignant potential in colon cancer cell line

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Key words

Atonal homolog 1, cancer stem cells, colitis-associated cancer, inflammatory bowel disease, signet ring cell

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Patients with inflammatory bowel disease (IBD) have an increased risk of developing colitis-associated colorectal cancer (CAC). CAC cells often develop chemoresistance, resulting in a poorer prognosis than that of sporadic colorectal cancer (CRC). The mechanism by which CAC enhances malignant potential remains unknown. We have previously reported that the proteasomal degradation of the transcription factor Atonal homolog 1 (Atoh1) protein results in the non-mucinous form of CRC. It also remains unknown whether Atoh1 protein is expressed in CAC. Therefore, in the present study, we investigated whether Atoh1 protein stabilizes in CAC. Consequently, the treatment with TNF-α stabilized Atoh1 protein through the inactivation of GSK-3β via Akt, resulting in the mucinous form of CRC cell lines. Atoh1 protein also enriched cancer stem cells with upregulated Lgr5 expression and cells in G0/G1 cell cycle phase, resulting in both the chemoresistance to 5-fluorouracil and oxaliplatin and the promotion of cell migration. Immunofluorescence of the human mucinous CAC specimens showed the accumulation of NF-κB p65 at nuclei with the expression of Atoh1 in mucinous cancer. In conclusion, the inflammation associated with carcinogenesis may preserve the differentiation system of intestinal epithelial cell (IEC), resulting in the acquisition of both the mucinous phenotype and high malignant potential associated with the enrichment of cancer stem cell.

Patients with inflammatory bowel disease (IBD) generally present with ulcerative colitis (UC) and Crohn's disease (CD); these patients have an increased risk of developing colitis-associated colorectal cancer (CAC), particularly 8–10 years from the diagnosis of IBD.[1] The risk of colorectal cancer (CRC) is 20-fold to 30-fold higher in patients with UC than that in the general population.[2] In Asia, including Japan, the number or patients with IBD is increasing; hence, it is likely that the number of patients in Asia with CAC will rise as well.[3] In particular, we need to be aware of the higher malignant potential of CAC than that of CRC. It is difficult to find CAC in its early stages by endoscopy because CAC develops towards the sub-mucosa as flat lesions.[4] Consequently, the typically advanced stage of CAC at diagnosis shortens the prognosis of the patients.[5] Moreover, mucinous carcinoma (MC) and signet ring cell carcinoma (SRCC) are typical pathological findings in CAC,[6] suggesting that the characterization of CAC may be fundamentally different from CRC. However, little is known about why CAC has high malignant potential and how it frequently generates MC and SRCC. First, we focused on the pathological characteristics for maintaining secretory capacity in MC and SRCC. One of the most important genes for cell formation is the basic helix-loop-helix transcription factor Atonal homolog 1 (Atoh1), which is essential for differentiation towards secretory lineages in the small and large intestines.[7–9] Therefore, we have demonstrated that the Atoh1 protein is actively degraded in sporadic CRC through the ubiquitin–proteasomal system, resulting in the disappearance of the mucinous form of CRC.[10] In addition, the Atoh1 and β-catenin proteins are reciprocally regulated by glycogen synthase kinase-3β (GSK-3β) in Wnt signaling.[11] In contrast, it has been reported that the Atoh1 protein is expressed in MC and SRCC,[12] both of which have secretory capacity, suggesting that Atoh1 may regulate the mucinous phenotype of colon

cancer. We have also demonstrated that stable expression of a mutant Atoh1 protein reconstituted with alanine in place of five serine residues in colon cancer induced not only differentiation but also cancer stemness and chemoresistance,[13] resulting in a high malignant potential of CRC; however, it remains unknown as to how Atoh1 protein is permanently expressed in MC and SRCC. Therefore, in this study we aimed to investigate the mechanism of the stabilization of Atoh1 protein in CAC. TNF-α stabilizes Atoh1 protein through the inhibition of GSK3 activity via Akt phosphorylation. In the present study, Atoh1 expression in the CRC cell line was associated with high malignant potential, as well as chemoresistance, the enrichment of cancer stem cells and cell migration. Atoh1 protein expression in the patients with CAC with NF-κB signal activation resulted in the mucinous form of CRC.

Materials and Methods

Cell culture and chemicals. Sporadic human colon cancer-derived DLD1 cells were cultured as described previously.[13] Plasmid DNA was transfected by lentivirus infection, as described previously.[14] Blasticidin (7.5 μg/mL, Invitrogen, Carlsbad, CA, USA) was used only in the selection of transfected cells. Blasticidin was excluded during the treatment with reagents. Cells were cultured with or without TNF-α (50 ng/mL Peprotech, Rocky Hill, NJ, USA), lipopolysaccharides (LPS) (1 μg/mL; Sigma-Aldrich, St. Louis, MO, USA) or flagellin (100 ng/mL; InvivoGen, San Diego, CA, USA). 5-Fluorouracil (5-FU; 20 μM [Tocris Cookson, Ellisville, MI, USA]) and oxaliplatin (L-OHP; 100 μM [Tocris Cookson]) were used for evaluating chemoresistance.

Plasmids. The mCherry-Atoh1 vector was generated by inserting Atoh1 gene into the mCherry DNA template PG27188 (DNA 2.0; Menlo Park, CA, USA). The Atoh1-lentivirus vector was generated by inserting the PCR-amplified mCherry-Atoh1 gene into pLenti 6.4 with CMV promoter (Invitrogen). The S/G2/M-green-lentivirus vector was generated by inserting the PCR-amplified pFucci-S/G2/M-green DNA sequence into pLenti 6.4, as described previously.[13]

Human samples. Human colonic specimens were obtained from patients with CAC associated with UC (n = 5), CD (n = 1) and the patients with sporadic CRC (n = 5) who underwent colectomy. The information for patients with CAC is as follows. Average age 40.67 years (35–53 years); male: female 6:0; average duration of IBD at the onset of CAC 18.8 years (8–36 years); depth of CAC invasion MP n = 1, SS n = 2, A n = 3; and cancer stages I n = 1, IIA n = 3, IIIC n = 1, IV A n = 1. These experiments were approved by the Tokyo Medical and Dental University Hospital Ethics Committee on Human Subjects.

Quantitative real-time PCR. Total RNA was isolated using an RNeasy Mini Kit (Qiagen, Venlo, Netherlands) according to the procedure manual. Complementary DNA synthesis was performed as described previously.[13] One microliter of complementary DNA was amplified using QuantiTect SYBR Green PCR Kits (Qiagen) in a 20 μL reaction. The primer sequences are summarized in Table S1. In all experiments, the expression in LS174T cells (mucinous phenotype of colon cancer cell line) was used as standard. The amount of mRNA was normalized by β-actin.

Western blot analysis. Cells were extracted with 1% SDS, containing radioimmunoprecipitation assay buffer as described previously,[10] or 1% NP-40, containing 10 mM Tris-HCl buffer, 150 mM NaCl, 1 mM EGTA, 1.5 mM MgCl2, 100 mM NaF and complete protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany) for the detection of

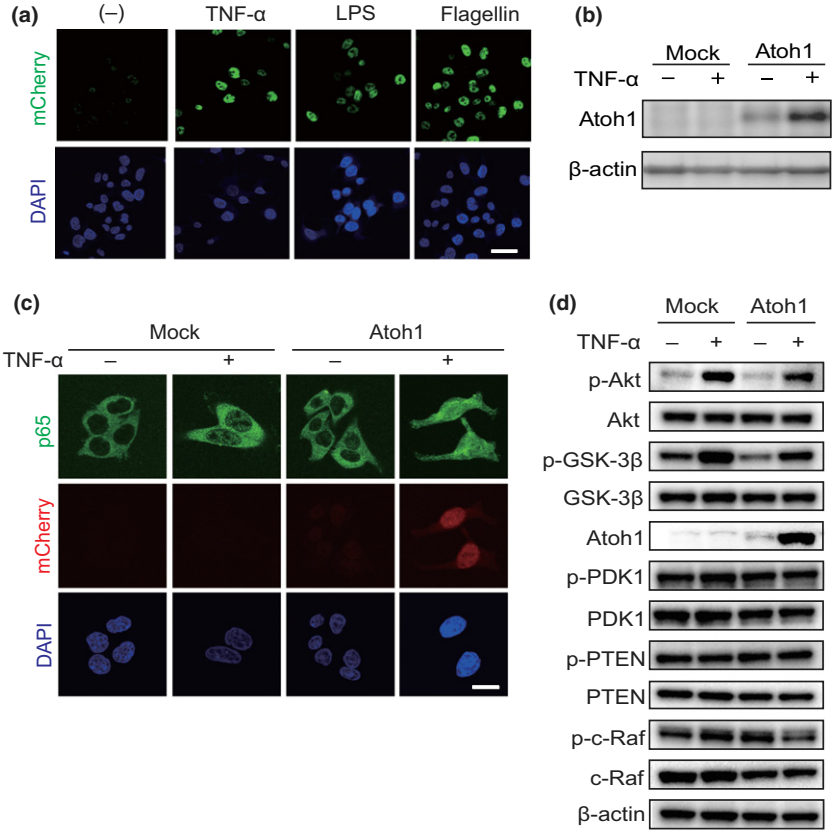

Fig. 1. Atonal homolog 1 (Atoh1) protein is stabilized in colon cancer cells by NF-κB signal activation. (a) mCherry-Atoh1 or mock DNA was transfected into DLD1 cells (Atoh1-DLD1 cells and mock-DLD1 cells). After 4 weeks in culture with inflammatory materials, the expression of mCherry-Atoh1 protein was determined by immunofluorescence using mCherry antibody and Alexa 488 antibody (green fluorescence). mCherry protein was stably expressed in the nuclei of Atoh1-DLD1 cells cultured with TNF-α, lipopolysaccharides (LPS) and flagellin. Scale bar, 50 μm. (b) Western blot analysis using Atoh1 antibody showed the expression of Atoh1 protein in Atoh1-DLD1 cells with TNF-α for 4 weeks. Endogenous Atoh1 protein was not detected in mock-DLD1 cells with TNF-α because the endogenous Atoh1 gene was not expressed in DLD1 cells. (c) Immunofluorescence staining showed nuclear localization of NF-κB p65 by treatment with TNF-α for 20 weeks. mCherry fluorescence was shown in the nuclei of only Atoh1-DLD1 cells with TNF-α, indicating the stable expression of Atoh1. Scale bar, 20 μm. (d) Western blot analysis of DLD1 cells during treatment with TNF-α for 20 weeks.

phosphorylated protein. Preparations of 50 or 100 μg of proteins were separated in NuPAGE 4–12% gradient Bis-Tris gel (Invitrogen) and transferred to membranes according to standard procedures. The membranes were immunoblotted with primary antibodies, including anti-Atoh1 (originally generated as previously described),[10] anti-phospho-Akt (Ser473) (Cell Signaling, Beverly, MA, USA), anti-Akt (Cell Signaling), anti-phospho-GSK-3β (Ser9) (Cell Signaling), anti-GSK-3β (Cell Signaling), anti-phospho-PDK1 (Ser241) (Cell Signaling), anti-PDK1 (Cell Signaling), anti-phospho-PTEN (Ser380) (Cell Signaling), anti-PTEN (Cell Signaling), anti-phospho-c-Raf (Ser259) (Cell Signaling), anti-c-Raf (Cell Signaling), anti-cleaved caspase-3 (Asp175) (Cell Signaling), anti-caspase-3 (Cell Signaling), anti-cleaved caspase-9 (Asp330) (Cell Signaling), anti-caspase-9 (Cell Signaling), anti-cleaved PARP (Asp214) (Cell Signaling), anti-PARP (Cell Signaling) and anti-β-actin (Sigma-Aldrich), before incubation with secondary antibodies.

The other material and methods are described in the supplementary material and methods (Data S1).

Results

Atonal homolog 1 protein is stabilized in colon cancer cells by NF-κB signal activation. To assess the stabilization of Atoh1 protein in colon cancer, we attempted to construct Atoh1 gene

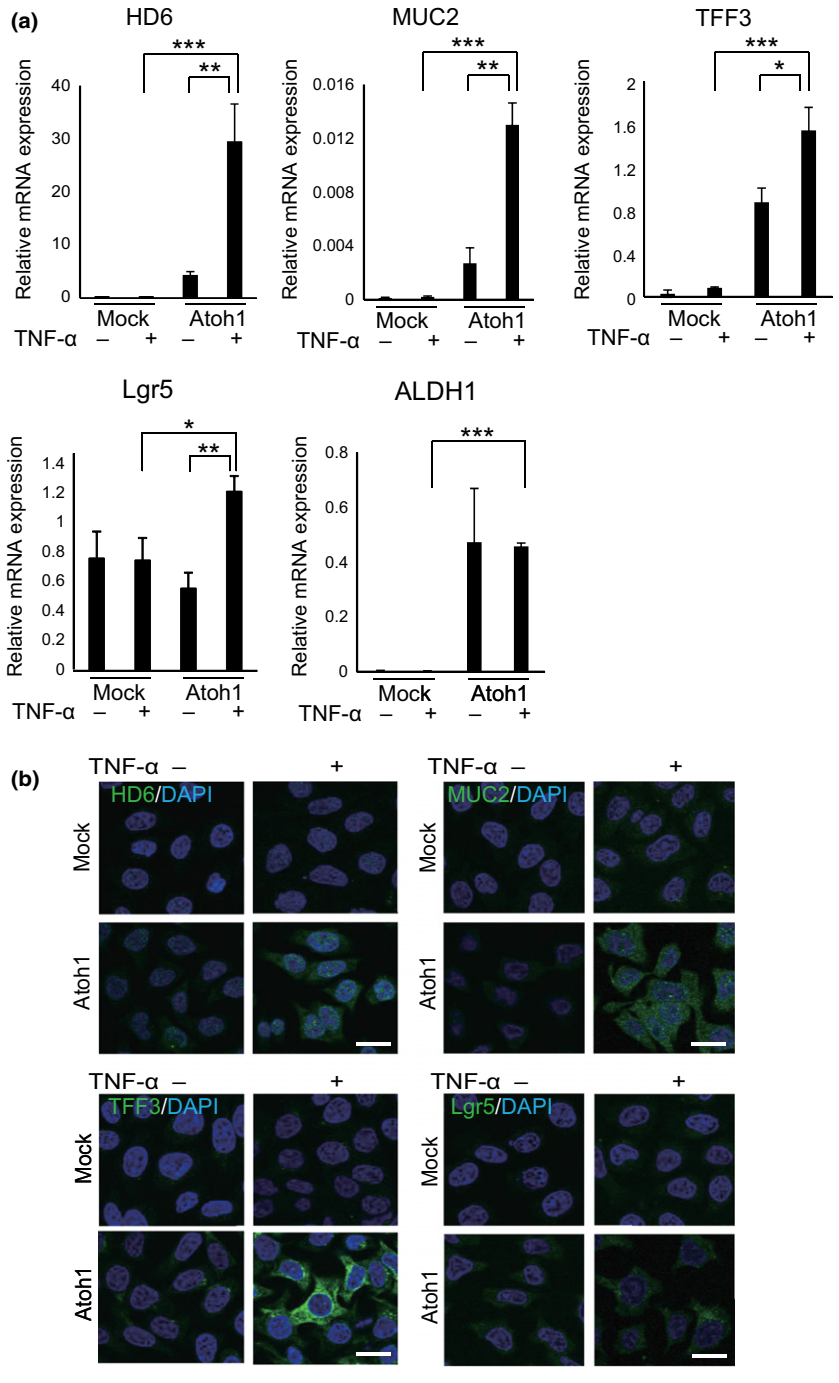

Fig. 2. Atonal homolog 1 (Atoh1) protein stabilized by TNF-α in colon cancer acquires mucinous secretion and upregulated Lgr5 expression. (a) HD6, MUC2 and TFF3 genes were significantly upregulated in Atoh1-DLD1 cells cultured with TNF-α for 20 weeks by RT-PCR. Lgr5 gene was also upregulated in Atoh1-DLD1 cells with TNF-α. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $n = 3$. (b) Immunofluorescence of HD6, MUC2, TFF3 and Lgr5 showed the expression of these proteins in Atoh1-DLD1 cells with TNF-α treatment for 20 weeks. Scale bar, 20 μm.

linked to mCherry to visualize Atoh1 protein expression. Our previous study indicated that Atoh1 protein was not detected by Atoh1 gene transfection alone in DLD1 cells derived from human sporadic CRC, in which endogenous Atoh1 gene was not expressed.[13] Therefore, we tried various reagents related to inflammation to stabilize Atoh1 protein, resulting in the expression of Atoh1 protein in mCherry-Atoh1 gene-expressing DLD1 cells (Fig. 1a). Among these reagents, TNF-α is one of the most important proinflammatory cytokines, with a central role in intestinal inflammation.[15] Antibodies targeting TNF-α have provided the most successful approach in the clinical management of IBD.[16] Therefore, we focused on the role of TNF-α in Atoh1 protein stabilization. We then confirmed the stabilization of Atoh1 protein by TNF-α (Fig. 1b). To assess the effect of long-term treatment with TNF-α on cancer cells, we added TNF-α to each cell culture for 20 weeks. Fluorescence analysis showed the stable expression of Atoh1 protein in the nuclei of DLD1 cells with NF-κB signal activation by TNF-α (Fig. 1c). To assess the regulation of Atoh1 protein stabilization through the phosphorylation via GSK-3β, we investigated the activation of GSK-3β by its own phosphorylation by Akt. Western blotting revealed that TNF-α treatment induced the inactivation of GSK-3β function through the phosphorylation of GSK-3β. Because Akt is well known as an upstream mediator in the signal cascade

Table 1. Stable expression of Atonal homolog 1 protein by TNF-α enriches cancer stem cells

Number of cells	Number of spheroids			
	Mock		Atoh1	
TNF-α	–	+	–	+
5000	359.3	381	361.3	411.3
2500	186.7	182.3	188	221.3
1000	58.3	68	72	81.7
500	29.7	31.7	31	42.3
Spheroid-forming frequency	1/49392	1/47148	1/48210	1/41128
(95% confidence interval)	(1/51671–1/47214)	(1/49274–1/45113)	(1/50408–1/46108)	(1/42864–1/39462)
P-value (*vs* mock, TNF-α –)	–	0.147	0.452	3.62×10^{-9}
P-value (*vs* Atoh1, TNF-α +)	3.62×10^{-9}	8.54×10^{-6}	2.6×10^{-7}	–

Cells were treated with TNF-α for 20 weeks. Various concentrations of DLD1 cells (5000, 2500, 1000 and 500 cells/well) were cultured. After 7 days, the number of spheroids was counted. The confidence interval (95%) for spheroid-forming frequency was calculated using a software application for limiting dilution analysis, ELDA. $n = 3$; –, not applicable.

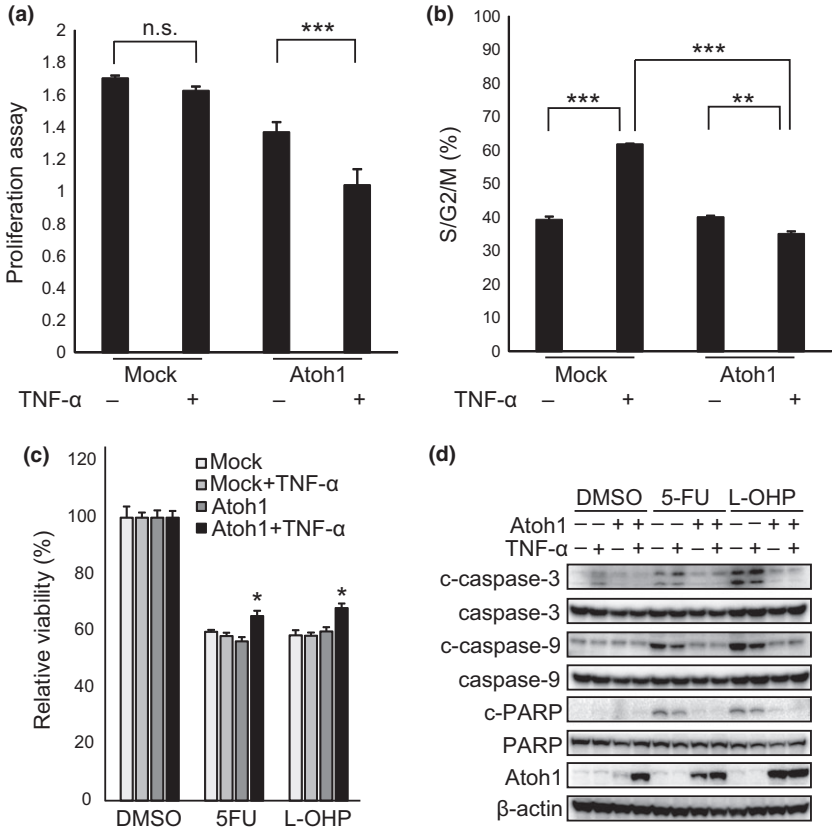

Fig. 3. Stable expression of Atonal homolog 1 (Atoh1) protein by TNF-α leads to higher malignant potential. (a) Proliferation assay revealed that Atoh1-DLD1 cells with TNF-α for 20 weeks showed slow growth. ***$p < 0.001$, $n = 6$. (b) S/G2/M phase of cells were visualized using the Fucci system. FACS analysis showed that TNF-α for 20 weeks decreased the number of Atoh1-DLD1 cells in the S/G2/M phase, whereas TNF-α increased the number of mock-DLD1 cells in the S/G2/M phase. **$p < 0.01$, ***$p < 0.001$, $n = 3$. (c) After 20 weeks culture with TNF-α, DLD1 cells were treated with 5-Fluorouracil (5-FU; 20 μM), oxaliplatin (L-OHP; 100 μM) or DMSO for 48 h to examine the chemoresistance. MTS assay showed that the reduction of cells in Atoh1-DLD1 cells with TNF-α by treatment with 5-FU or L-OHP was less than that of others. *$p < 0.001$, $n = 6$. (d) After treatment with 5-FU or L-OHP for 48 h, apoptosis signal was assessed by western blot. The stabilization of the Atoh1 protein by TNF-α increased the resistance to apoptosis.

involved in GSK-3 phosphorylation,[17] Akt activation was also assessed. Our results showed that the phosphorylation of Akt was induced by TNF-α. However, TNF-α did not affect any other phosphorylation enzymes in the signal cascade of GSK-3β phosphorylation (Fig. 1d).

Atonal homolog 1 protein stabilized by TNF-α in colon cancer acquires mucinous secretion and leucine-rich repeat-containing G-protein coupled receptor 5 expression. We performed continuous treatment with TNF-α in DLD1 cells for 20 weeks, resulting in the stable expression of Atoh1 protein. The stable expression of Atoh1 protein by TNF-α resulted in the induction of secretory phenotypic genes and proteins, such as human α-defensin 6 (HD6), mucin 2 (MUC2) and trefoil factor 3 (TFF3) (Fig. 2). The expression in LS174T cells, which are the mucinous colon cancer cell line, was used as the standard in all PCR experiments because the value of gene expression in LS174T cells was defined as 1. Thus, Atoh1 protein-expressing cells were generated for the first time in colon cancer, resulting in the acquisition of a mucinous phenotype, although MUC2 expression in Atoh1-expressing cells was lower than that in LS174T cells. Moreover, the expression of the leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) gene, but not the ALDH1 gene, was increased in Atoh1 protein-expressing cells induced by TNF-α (Fig. 2).

Stable expression of Atoh1 protein by TNF-α enriches cancer stem cells. To further analyze the functional role of Atoh1 protein in colon cancer cells, we examined whether the Atoh1 protein affected malignant potential. Because we have previously reported that the stable expression of mutant Atoh1 protein enhances cancer stem cells with Lgr5 induction,[13] we assessed whether the wild type Atoh1 protein stabilized by TNF-α enriched cancer stem cells using an *in vitro* spheroid assay. Extreme limiting dilution analysis (ELDA) showed that the stable expression of Atoh1 protein by TNF-α enriched the spheroids (Table 1), supporting the suggestion of the enrichment of cancer stem cells by Atoh1 protein stabilization.

Stable expression of Atoh1 protein by TNF-α leads to higher malignant potential. As expected from the results of a previous study using mutant Atoh1, Atoh1 protein stabilization by TNF-α suppressed cell proliferation in all colon cancer cells, whereas the treatment with TNF-α alone in mock DNA-expressing DLD1 cells did not change cell proliferation (Fig. 3a). As the cell cycle may affect cell proliferation in cells expressing Atoh1 protein, we attempted to visualize the cell cycle using a fluorescent ubiquitination-based cell cycle indicator (Fucci) system in which cells in the S/G2/M phase were marked by Azami-Green 1 fused with geminin. FACS analysis showed the decrease of the S/G2/M phase in Atoh1-expressing cells, indicating enrichment of the cells in G0/G1 phase. In contrast, the treatment with TNF-α in mock-expressing cells yielded enriched cells in S/G2/M phase (Fig. 3b). To confirm the malignant potential induced by Atoh1 protein in colon cancer cells, we assessed whether the Atoh1 protein conferred chemoresistance. The cells in which Atoh1 protein was stabilized by TNF-α were more resistant to 5-FU and L-OHP than mock-transfected cells and Atoh1 transfected cells alone (Fig. 3c). Apoptosis signals were also suppressed in Atoh1 transgene cells (Fig. 3d).

Stable expression of Atoh1 protein by TNF-α promotes cell migration. We assessed whether Atoh1 promotes cell migration. Atoh1 protein stabilization by TNF-α promoted cell migration, whereas the treatment with TNF-α alone in mock DNA-expressing DLD1 cells did not change cell migration (Fig. 4a,b).

The human zinc transcription factor (ZEB1), one of the major regulators in TGF-β-induced epithelial–mesenchymal transition (EMT),[18] was significantly upregulated only in Atoh1-expressing cells treated with TNF-α (Fig. 4c). Although

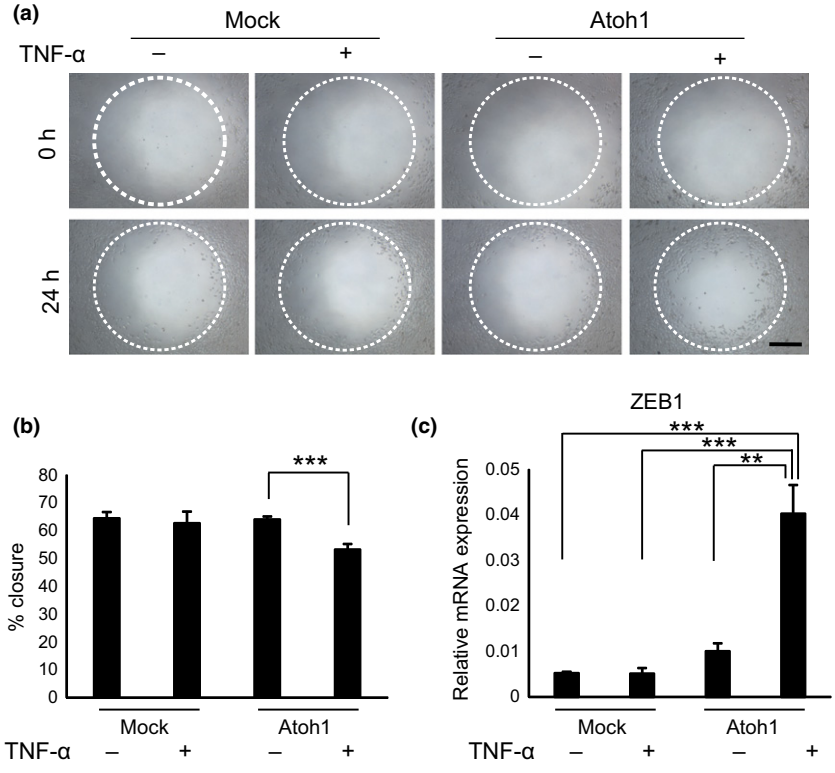

Fig. 4. Stable expression of Atonal homolog 1 (Atoh1) protein by TNF-α promotes cell migration. (a) Migration assay showed that the vacant area was occupied by a greater number of Atoh1-DLD1 cells cultured with TNF-α for 20 weeks than the others. Scale bar, 500 μm. (b) The ratio of the remaining vacant area is shown. The vacant area of Atoh1-DLD1 cells was smaller than that of others at 24 h after the cells were seeded. $^{***}P < 0.001$, $n = 6$. (c) The expression of ZEB1 in DLD1 cells was analyzed by RT-PCR. ZEB1 gene was significantly upregulated in Atoh1-DLD1 cells with TNF-α treatment for 20 weeks. $^{**}P < 0.01$, $^{***}P < 0.001$, $n = 3$.

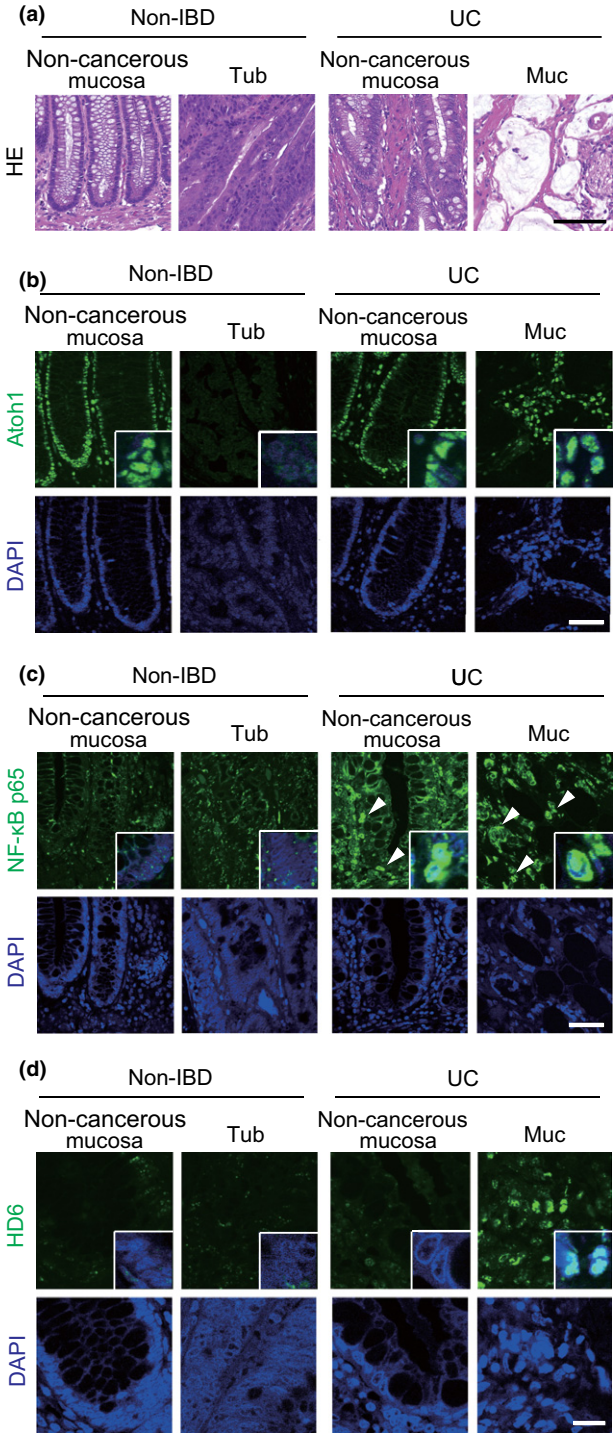

Fig. 5. Atonal homolog 1 (Atoh1) protein was expressed in colitis-associated colorectal cancer (CAC) with NF-κB signal activation. (a) HE-staining of colonic specimen taken from patients with colorectal cancer (CRC) or CAC with ulcerative colitis (UC). Scale bar, 100 μm. Muc, mucinous adenocarcinoma; Tub, tubular adenocarcinoma. (b) Immunofluorescence staining of Atoh1 showed Atoh1 protein expression in mucinous carcinoma (MC) of CAC. Scale bar, 50 μm. Lower right frame shows a high-magnified image merged with DAPI. (c) Immunofluorescence analysis of NF-κB p65. The nuclear localization of p65 is shown in both the inflamed and cancer region of colon in patients with UC. The white arrow head indicates a cell with nuclear localization of p65. Scale bar, 50 μm. The lower right frame shows a high-magnified image merged with DAPI. (d) Immunofluorescence staining of HD6 showed the expression of HD6 only in CAC of the colon. Scale bar, 20 μm. The lower right frame shows a high-magnified image merged with DAPI.

ZEB2 is also involved in the TGF-β-induced EMT, ZEB2 was not detected by RT-PCR in several colon cancer cell lines. Moreover, ZEB2 was not upregulated by Atoh1 protein expression (data not shown).

Atoh1 protein was expressed in colitis-associated cancer with NF-κB signal activation. We assessed the expression of Atoh1 in CAC in the patients with UC. Immunofluorescence analysis showed that Atoh1 protein was expressed in mucinous carcinoma (MC) lesions of CAC, whereas Atoh1 was not detected in tubular adenocarcinoma in CRC (Fig. 5a,b). NF-κB p65 was located, in part, at the nuclei of the intestinal epithelial cell in patients with UC. NF-κB p65 was also located at the nuclei of the MC in CAC, whereas NF-κB p65 was located in the cytoplasm of both noncancerous and cancerous lesions in patients with CRC (Fig. 5c). We also assessed the expression of human α-defensin 6 (HD6) in CAC. HD6 was not detected in the colon because HD6 was secreted from Paneth cells in the small intestine. HD6 was expressed only in the mucinous form of CAC (Fig. 5d). Moreover, HD6 was highly expressed in the cancer cells of all CAC patients (n = 6) (Fig. S1).

Discussion

The results of this study revealed that Atoh1 protein could be stably expressed in colon cancer cells by TNF-α treatment. We also demonstrated that Atoh1 protein stabilization induced both a cancer stem cell phenotype and a mucinous phenotype, resulting in the acquisition of chemoresistance. Our previous study showed that the mutated Atoh1 protein could be stably expressed while maintaining transcriptional activity in colon cancer cells, mimicking the phenotype of MC and SRCC.[13] However, the study did not reveal how Atoh1 protein was stabilized in MC and SRCC. Consistent with our previous study, transfection of Atoh1 gene alone could not induce the mucinous phenotypic gene expression because of proteasomal degradation of Atoh1 protein. In contrast, the treatment with TNF-α induced the stable expression of Atoh1 protein that resulted in the acquisition of the characteristic of MC and SRCC. The stabilization of Atoh1 protein has been shown to be regulated by the enzyme activity of GSK-3β.[10] We assessed the relationship between TNF-α and GSK-3β. It appeared that TNF-α suppressed the enzyme activity of GSK-3β via Akt phosphorylation of GSK-3β. Although it is well known that Akt induces the inactivation of GSK-3β via PI3K pathway,[19] there are only a few reports concerning the TNF-α activation of Akt through its phosphorylation.[20] The other enzymes, such as PDK1 and PTEN, were not affected by TNF-α; this suggested that TNF-α activates Akt via an unknown pathway other than the PI3K pathway. It may be important to elucidate the pathway between TNF-α and Akt for the formation of pathological type in CAC. Other NF-κB signal stimulating factors,[21] such as LPS and flagellin, also stabilized Atoh1 protein in DLD1 cells, suggesting that NF-κB signaling via Toll-like receptors may affect the activity of GSK-3β. It has been reported that NF-κB signaling was essential for the carcinogenesis in CAC model mice.[22] This suggests that NF-κB may have a role in the regulation of both malignant transformation and the pathological type in CAC through stabilizing Atoh1 protein during the carcinogenesis in the IBD patients. Of note, TNF-α blockers, such as Infliximab and Adalimumab, have already been used for the treatment of IBD all over the world.[23] Treatment with TNF-α blockers for IBD may be useful for not only the inhibition of carcinogenesis but also for that of malignant potential in CAC.

Another finding of particular interest is that Atoh1 protein in colon cancer conferred not only a mucinous phenotype but also a cancer stem cell phenotype, as indicated by ELDA, Lgr5 expression, cell cycle arrest, migration and chemoresistance. In our previous study, the expression of mutant Atoh1 protein in colon cancer revealed similar results, suggesting that Atoh1 plays crucial roles in the formation of MC and SRCC in CAC.

Atonal homolog 1 protein was detected in cells treated with 5-FU and L-OHP, which induced the suppression of GSK-3α kinase activity, as previously described.[13] The apoptosis signal by 5-FU and L-OHP incubation for 48 h was suppressed in the Atoh1 gene transfected cells with or without TNF-α treatment. Because the Atoh1 transfected cells without TNF-α treatment may be initially sensitive to anti-cancer drugs, the total number of Atoh1 transfected cells without TNF-α after 48 h of incubation with these drugs was less than that of Atoh1 transfected cells with TNF-α. We speculated that the expression of Atoh1 in cancer cells promotes their differentiation, resulting in lower malignant potential. However, the present study revealed that the expression of Atoh1 promoted higher malignant potential of cancer. When Atoh1 induced the differentiation of IEC in normal mucosa, Wnt signal was blocked, resulting in the degradation of β-catenin protein. However, the stabilization of Atoh1 protein in cancer cells resulted in the colocalization of β-catenin protein that was stabilized by the aberrant Wnt signaling. In this study, we also showed that the expression of Lgr5, which can be induced by β-catenin,[24,25] was further induced by Atoh1, suggesting the cooperation between Atoh1 and β-catenin increases the malignant potential in MC and SRCC. Because the possibility of an artifact as a result of experimental conditions in the overexpression of Atoh1 exists, the function of endogenous Atoh1 protein should be analyzed in future.

An additional finding, of particular interest, of this study is that HD6 may be a specific marker for CAC in the colon. HD6 is strictly expressed in Paneth cells at the bottom of the crypt in the small intestine as antibacterial protein.[26] The expression of HD6 and HD5, which are the members of α-defensin,[27] is regulated by β-catenin by binding to TCF4 motif sequence on the promoter region of HD6 and HD5. We recently found that Atoh1 also regulated the expression of HD6, but it did not regulate that of HD5, by binding to the E-box sequence on the promoter region of HD6 in cooperation with β-catenin protein in Paneth cells (unpublished data), indicating that Atoh1 and β-catenin-expressing colon cancer may specifically express HD6. Because it is very difficult to detect CAC in the early stages, the expression of HD6 in a colonic biopsy specimen may be useful in diagnosing CAC. A large-scale study for the expression of HD6 in CAC should be carried out to confirm its specificity.

In conclusion, Atoh1 stabilization by TNF-α mimics MC and SRCC in CAC, with both stem cell and differentiation phenotypes resulting in chemoresistance. Recently, we established a method for the primary culture of colon epithelial cells as organoids for long periods.[28] The establishment of an *in vitro* carcinogenesis model of CAC using this culture method may enable us to observe the malignant transformation from benign epithelial cells.

Acknowledgments

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Disclosure Statement

The authors have no conflict of interest to declare.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Data S1. Supplementary materials and methods.

Fig. S1. High expression of HD6 in colitis-associated cancer (CAC).

Table S1. Primer lists for the present study are summarized.