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The acquisition of malignant potential in colon cancer is regulated by the stabilization of Atonal homolog 1 protein

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ABSTRACT

The transcription factor Atonal homolog 1 (Atoh1) plays crucial roles in the differentiation of intestinal epithelium cells. Although we have reported that the Atoh1 protein was degraded in colon cancer by aberrant Wnt signaling, a recent study has indicated that the Atoh1 protein is expressed in mucinous colon cancer (MC) and signet ring cell carcinoma (SRCC). However, the roles of the Atoh1 protein in MC are unknown. To mimic MC, a mutated Atoh1 protein was stably expressed in undifferentiated colon cancer cells. Microarray analysis revealed the acquisition of not only the differentiated cell form, but also malignant potential by Atoh1 protein stabilization. In particular, Atoh1 enhanced Wnt signaling, resulting in the induction of Lgr5 as a representative stem cell marker with the enrichment of cancer stem cells. Moreover, the fluorescent ubiquitination-based cell cycle indicator system with time-lapse live imaging demonstrated cell cycle arrest in the G0/G1 phase by Atoh1 protein stabilization. In conclusion, the Atoh1 protein regulates malignant potential rather than the differentiation phenotype of MC, suggesting the mechanism by which MC and SRCC are more malignant than non-mucinous adenocarcinoma.

1. Introduction

Pathological differentiation has been one of the most reliable histological criterion to predict the effectiveness of chemotherapy and the prognosis of colon cancer. Many reports support the idea that pathological differentiation is closely related to the progression and chemoresistance of a cancer [1]. However, "pathological differentiation" has often been confused with cell differentiation because the classification of pathological differentiation is based on the ductal formation of cancer cells. Interestingly, cancer cells that maintain the differentiated form such as mucinous carcinoma (MC) and signet ring cell carcinoma (SRCC) have often been classified as undifferentiated tumors on the basis of pathological findings. Nevertheless, the cell differentiation mechanism of colon cancer has not been investigated. One of the most important genes for cell formation is the basic helix–loop–helix (bHLH) transcription factor, Atonal homolog 1 (Atoh1), which is essential for differentiation toward secretory lineages in the small and large intestine [2]. Previous reports have suggested that the Atoh1 gene was suppressed by Wnt signaling in some colon cancers [3]. Moreover, we have demonstrated that the Atoh1 protein was actively degraded in colon cancer by the ubiquitin proteasomal system resulting in the disappearance of the Atoh1 protein in colon cancer despite Atoh1 gene expression [4]. Collectively, the deletion of adenomatous polyposis coli (APC) in colon carcinogenesis causes Atoh1 protein degradation by switching it to become the target of glycogen synthase kinase 3β GSK3β rather than β-catenin, resulting in maintenance of the undifferentiated cellular state [4]. On the other hand, it has been reported that the Atoh1 protein was expressed in MC and SRCC, both of which have secretory capacity [5]. It is notable that MC and SRCC are often classified as poorly differentiated tumors from pathological findings, resulting in a poorer

Abbreviations: Atoh1, Atonal homolog 1; MC, mucinous colon cancer; SRCC, signet ring cell carcinoma; GSK3, glycogen synthase kinase 3; Lgr5, Leucine-rich repeat-containing G-protein coupled receptor 5; IEC, intestinal epithelial cells; MUC2, mucin 2; TFF3, trefoil factor 3; HD6, human defensin 6; TCF4, T-cell factor 4; RT-PCR, reverse-transcription polymerase chain reaction; RLU, relative light units.

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prognosis than sporadic colon cancer. The relationship between pathological differentiation and cellular differentiation in colon cancer has not been clarified. Moreover, the significance of Atoh1 expression in cancer cells for malignant potential is controversial. It has been reported that the deletion of Atoh1 prevents cerebellar neoplasia in a mouse model of medulloblastoma in brain [6], suggesting that Atoh1 acts as a tumor accelerator. In contrast, Atoh1 is reported to be a tumor suppressor gene in Merkel cell carcinoma and colon cancer because Atoh1 suppressed cell proliferation [7]. However, there is no evidence except for the suppression of cell proliferation to confirm that in colon cancer Atoh1 acts as a tumor suppressor. Moreover, previous studies of Atoh1 function in colon cancer have analyzed the Atoh1 gene expression; however, because the expressed Atoh1 protein is degraded, its function has not been elucidated in detail.

In this study, we investigated the functions of Atoh1 in colon cancer to clarify the definition of cancer cell differentiation. We demonstrate that stable expression of the Atoh1 protein in colon cancer induces not only differentiation, but also the promotion of the malignant potential of colon cancer.

2. Materials and methods

2.1. Cell culture and chemicals

Sporadic human colon cancer-derived SW480, DLD1 cells and human embryonic kidney-derived 293T cells were cultured as described previously [8] Plasmid DNA was transfected as described previously [8]. Lentivirus infection was performed according to the manufacturer's protocols. The infected cell lines were supplemented with Blasticidin (7.5 μg/ml, Invitrogen Carlsbad, CA, USA) during maintenance. Oxaliplatin (L-OHP) was used (Tocris Cookson, Ellisville, MI, USA) for evaluating chemoresistance.

2.2. 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 mutant (5SA-Atoh1) was constructed by PCR-mediated mutagenesis in which five serine residues, TCC (160–162) and AGC (172–174, 328–330, 340–342, 352–354), were replaced with alanine residue GCC. The Atoh1-lentivirus vector was generated by inserting the PCR-amplified mCherry-Atoh1 gene or mCherry-5SA-Atoh1 into pLenti 6.4 (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 (Invitrogen). The lentivirus was generated according to the procedure manual. 5′ Lgr5 reporter plasmid was generated by cloning a 1000 bp sequence 5′of the human Lgr5 gene into a pGL4 basic vector (Promega, Madison, WI). The promoter region of Lgr5 was gradually shortened by 200–1000 bp was generated into a pGL4 basic vector. Polymerase chain reaction-mediated mutagenesis was used to construct internal deletion mutants of the 5′ Lgr5 reporter plasmid in which the following base pair sets −300 to −310, −310 to −320, −320 to −330, −330 to −340, and −340 to −350 were deleted separately.

2.3. Quantitative real-time PCR

Total RNA was isolated with TRIzol® reagent (Invitrogen) according to the manufacturer's instructions as described previously [8]. The primer sequences in this study are summarized in Supplementary Table S1. In all examinations, the expression in LS174T cells (mucinous phenotype colon cancer cell line) was used as standard.

2.4. Western blot analysis

Cells were extracted with 1% sodium dodecyl sulfate (SDS)-containing radioimmunoprecipitation assay (RIPA) buffer as described previously [8]. The membranes were immunoblotted with anti-mCherry (Clontech, Mountain View, CA, USA) and anti-USF2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibodies before incubation with secondary antibodies.

2.5. Immunofluorescence analysis

The cells were fixed as described previously [8]. The antibodies used were anti-human MUC2 (Ccp58; Santa Cruz Biotechnology), anti-human TFF3 (ab57752; Abcam, Cambridge, UK) and anti-human Lgr5 (TA301323; OriGene, Rockville, MD, USA). Anti-mouse IgG Alexa Fluor® 594 or Alexa Fluor® 488 (Invitrogen) were used as the secondary antibody. Cells were mounted with VectaShield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and visualized by confocal laser fluorescent microscopy (BZ-8000 [Keyence, Tokyo] and FLUOVIEW FV10i [Olympus, Tokyo]).

2.6. MTS assay

1×10^4 DLD1 cells were cultured in a 96-well tissue culture plate at 37 °C, and 5% CO_2. After a 48-h incubation period in the presence or absence of oxaliplatin, CellTiter96® Aqueous One Solution was added (20 μl/well) and incubated for 1 h at 37 °C and 5% CO_2. The absorbances at 490 nm were measured with an ARVO™MX plate reader (Perkin Elmer, Boston, MA, USA). Background absorbances from medium-containing wells were subtracted from those of the sample wells.

2.7. Migration assay

The Oris™ Pro Cell Migration Assay kit (Platypus Technologies, LLC USA) was used. This assay is formatted for 96 well-plates and uses a non-toxic biocompatible gel to form a cell-free zone on cell culture surfaces. DLD1 cells (1×10^4) were seeded into 96-well plates and incubated for 1 h. Phase contrast images were taken for pre-migration reference. After 6 h of incubation, images were captured using phase contrast microscopy. The ratio of the vacant area between pre- and post-migration was analyzed.

2.8. Cell cycle assay and live cell imaging

Live imaging was performed on the DeltaVision system (Applied Precision, Washington, USA) incorporating a fluorescent microscope IX-71 (Olympus, Tokyo, Japan) using a 20× 0.75NA Olympus UPlanSApo objective. Differential interference contrast (DIC) and fluorescent images were acquired at 15-min intervals for 72 h. The data were processed using softWoRx® (Applied Precision). Maximum intensity projections of the time series were exported into QuickTime format for presentation as Supplementary movies. The ratio of cells in the S/G2/M phase was analyzed by FACS caliber to detect cells expressing S/G2/M green fluorescence.

2.9. Chromatin immunoprecipitation assay

A chromatin immunoprecipitation (ChIP) assay was performed as described previously with some modifications [9]. The primer sequences in this study are summarized in Supplementary Table S1.