RNA editing is a valuable biomarker for predicting carcinogenesis in ulcerative colitis

Kazutaka Takahashi[1], Kunitoshi Shigeyasu[#1], Yoshitaka Kondo[1], Kazuyoshi Gotoh[7], Shuya Yano[1], Yuzo Umeda[1], Toshihiro Inokuchi[4], Caiming Xu[3, 8], Kazuhiro Yoshida[1], Hibiki Umeda[1], Toshiaki Takahashi[1], Sho Takeda[1], Ryuichi Yoshida[1], Fuminori Teraishi[1], Hiroyuki Kishimoto[1], Yoshiko Mori[1, 6], Kazuhiro Noma[1], Yoshinaga Okugawa[2], Sakiko Hiraoka[4], Hiroyuki Michiue[5], Hiroshi Tazawa[1], Osamu Matsushita[7], Ajay Goel[3], Toshiyoshi Fujiwara[1]

#: Corresponding author

[1] Department of Gastroenterological Surgery, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan
[2] Department of Gastrointestinal and Pediatric Surgery, Division of Reparative Medicine, Institute of Life Sciences, Mie University Graduate School of Medicine, Mie, Japan
[3] Department of Molecular Diagnostics and Experimental Therapeutics, Beckman Research Institute, City of Hope Comprehensive Cancer Center, CA, USA
[4] Department of Gastroenterology and Hepatology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan
[5] Neutron Therapy Research Center, Okayama University, Okayama, Japan
[6] Department of Digestive Tract and General Surgery, Saitama Medical Center, Saitama Medical University, Saitama, Japan
[7] Department of Bacteriology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan
[8] Department of general surgery, First Affiliated Hospital of Dalian Medical University, Dalian, China.

Corresponding author: Kunitoshi Shigeyasu, M.D., Ph.D., Department of Gastroenterological Surgery, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences; 2-5-1 Shikata-cho, Kita-ku, Okayama, 700-8558, JAPAN; E-mail: gmd421045@s.okayama-u.ac.jp

Short title: RNA editing in ulcerative colitis

Keywords: RNA editing, ulcerative colitis, carcinogenesis, biomarker

Grant support: This study was supported by grants from Takeda Science Foundation, Mochida Memorial Foundation, and JSPS KAKENHI (20K17653) to KS. This study was also supported by grants from JSPS KAKENHI (20K22848, 22K16533) to ST, and JSPS KAKENHI (21K16422) to Y. Kondo.

Disclosures: The authors declare that they have no competing interests.

Abbreviations: ADAR: Adenosine deaminase acting on RNA, AZIN1: Antizyme inhibitor 1, CAN: Colitis-associated colorectal neoplasm, CRC: Colorectal cancer, GO: Gene Ontology, IFN: Interferon, IHC: Immunohistochemistry, LOH: Loss of heterozygosity, LPS: Lipopolysaccharide, MES: Mayo endoscopic score, RESS q-PCR: RNA editing site-specific quantitative PCR, ROC curve: Receiver operating characteristic curve, TLR4: Toll-like receptor 4, UC: Ulcerative colitis

Author Contributions: Conceived and designed experiments: KT, KS, YK, KG, SY, YU, KY, TT, KN, TO, OM; performed experiments: KT, HU, KG, contributed reagents, materials, and other analytical tools: KT, KS, TI, CX, RY, FT, HK, KY, YM, YO, SH, HM, ST, HT, AG, TF; wrote the manuscript: KT, KS.

ABSTRACT

Background and Aims: Ulcerative colitis (UC) can develop colitis-associated colorectal neoplasm (CAN). Adenine-to-inosine RNA editing, which is regulated by adenosine deaminase acting on RNA (ADAR), induces the posttranscriptional modification of critical oncogenes, including antizyme inhibitor 1 (AZIN1), leading to colorectal carcinogenesis. Therefore, we hypothesized that ADAR1 might be involved in the development of CAN in UC.

Methods: We systematically analyzed a cohort of 139 UC cases (40 acute phase, 73 remission phase, 26 CAN). The degree of inflammation was evaluated using the Mayo endoscopic score (MES).

Results: The type 1 IFN-related inflammation pathway was upregulated in the rectum of active UC, rectum of UC-CAN, and tumor site of UC-CAN patients. ADAR1 expression was upregulated in the entire colon of CAN cases, while it was down-regulated in non-CAN MES0 cases. ADAR1 expression in the rectum predicted the development of CAN better than p53 or β-catenin, with an area under the curve of 0.93. The high expression of ADAR1 and high AZIN1 RNA editing in UC was triggered by type 1 IFN stimulation from UC-specific microbiomes, such as *Fusobacterium* in vitro analyses. The induction of AZIN1 RNA editing by ADAR1, whose expression is promoted by *Fusobacterium*, may induce carcinogenesis in UC.

Conclusions: The risk of CAN can be evaluated by assessing ADAR1 expression in the rectum of MES0 UC patients, freeing UC patients from unnecessary colonoscopy and reducing their physical burden. RNA editing may be involved in UC carcinogenesis, and may be used to facilitate the prevention and treatment of CAN in UC.

INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory disease affecting the colon and rectum, whose incidence is rising worldwide. UC is a chronic inflammatory disease characterized by mucosal inflammation starting distally in the rectum, with continuous extension proximally for a variable distance [1], affecting mainly the mucosa and submucosa, that most commonly presents in adults under 40 years of age but may also affect children and those over 50 years of age. The pathogenesis of UC is multifactorial, involving genetic predisposition, epithelial barrier defects, dysregulated immune responses, and environmental factors [2]. While the disease is idiopathic, immunopathological mechanisms and psychological factors are thought to be involved in its genesis.

Patients with UC can develop colitis-associated colorectal neoplasm (CAN), including high-grade dysplasia and colorectal cancer (CRC) [1]. CAN accounts for one in six deaths in patients with UC [1,3]. Risk factors for the development of CAN include the extent of the disease (extensive colitis), duration of disease (>8 years), family history of CRC, high inflammation, and primary sclerosing cholangitis [4,5]. Therefore, it is crucial to properly select cases that require surveillance colonoscopy at appropriate intervals. However, the optimal adaptability and interval of surveillance colonoscopy has not yet been determined. Additionally, finding CAN in a background of inflammation is challenging.

Adenine-to-inosine RNA editing, which is regulated by adenosine deaminase acting on RNA (ADAR), was recently shown to induce the posttranscriptional modification of critical oncogenes, including antizyme inhibitor 1 (AZIN1), leading to the promotion of stemness and colorectal carcinogenesis [6]. ADAR1 is induced by the inflammatory cytokine type 1 interferon (IFN) [7]. Thus, we hypothesized that ADAR1 might be involved in CAN development in UC because CAN risk is related to inflammation [4]. Therefore, in this study, we focused on the induction of ADAR1 by the inflammatory

environment of UC and investigated its role in the development of CAN to better identify patients requiring a total colonoscopy.

METHODS

Patients and sample collection

This study examined colorectal epithelial specimens from 139 UC cases (40 in the acute phase, 73 in the remission phase, and 26 with CAN, **Supplementary Table 1**). These samples were obtained from biopsies taken during surgery, endoscopic mucosal resection, or surveillance endoscopy performed at Okayama University Hospital between 2011 and 2020. The degree of inflammation was evaluated using the Mayo endoscopic score (MES), which was developed to assess the efficacy and safety of oral 5-aminosalicylic acid therapy for UC and has been the most frequently adopted index in recent large-scale clinical trials [5]. Three independent experts performed the histopathological examinations, and a panel of endoscopic experts evaluated the endoscopy results. The Institutional Review Board of Okayama University Hospital approved the specimen collection and studies (1907-001, 1907-002). All participants provided written informed consent and willingness to donate their tissue samples for research.

Immunohistochemistry (IHC) analysis

Paraffin-embedded sections were deparaffinized using xylene and ethanol. Endogenous peroxidase activity was eliminated with H_2O_2. The following primary antibodies were used: ADAR1 (1:100, ab88574, Abcam, Cambridge, UK), Iba1 (1:2000, ab178846, Abcam), p53 (1:200, clone DO-7, cat #M7001, Dako, Carpinteria, CA, USA), and β-catenin (1:250, 610154, BD, Franklin Lakes, NJ, USA).

IHC staining interpretation

ADAR1 staining intensity was evaluated in the nucleus and cytoplasm by three independent investigators blinded to the nature of the specimens (**Supplemental Figure 1**). Iba1 was evaluated by measuring the percentage of stain-positive areas in the mucosal epithelial-stromal region using ImageJ [8], and the percentage was defined as the Iba1 index. β-catenin staining was evaluated in the cell membrane and nucleus. A membranous staining pattern observed in normal cells was considered negative, whereas increased cytoplasmic and nuclear staining was evaluated as positive. The percentage of nuclear-stained cells was semi-quantitatively analyzed and divided into two categories: low (<10% of cells) or high (>10% of cells). Wnt activation was defined in cases where more than 10% of cells showed positive nuclear staining [9]. The specimens were deemed positive for p53 if the epithelial cells exhibited intense brown nuclear staining in the entire tissue sample or segments thereof (more than 20% positive cells) [9].

RNA-Seq and analysis

The mRNA-seq of colorectal specimens were collected from normal rectum: 6 cases, the rectum of UC patients under acute inflammation state (UC Acute-rectum): 6 cases, tumoral tissues of UC patients with CAN (UC CAN-CAN): 6 cases, and the rectum of UC patients with CAN (UC CAN-rectum): 6 cases. The mRNA sequencing library construction was performed using the TruSeq RNA library kit (Los Angeles, CA). High-throughput sequencing was carried out at DNA Chip Research Inc. We further filtered reads by removing adapter sequences, reads with low-quality (<Q20), and reads with more than 5% unknown bases called (N). Finally, clean reads were mapped to the human genome (GRCh38)

using the HISAT2 (v2.2.1) package [10], and the mapped reads were assigned to features using the HTseq-count (v0.12.3) package [11]. GSEA 4.0.3 software (downloaded from https://www.gsea-msigdb.org/gsea/downlodas.jsp) was used to identify Gene Ontology (GO) terms in the c5 GO database (c5.all.v6.2.symbols) [12]. The enrichment analysis of GSEA was considered statistically significant when $P < 0.05$.

Cell culture

The HT29, RKO, and CACO-2 CRC cell lines were purchased from the Japanese Collection of Research Bioresources Cell Bank (JCRB Cell Bank, Tokyo, Japan). All cell lines were cultured according to the manufacturer's specifications and were tested and authenticated every few months using a panel of established genetic markers.

Small interfering RNA (siRNA) transfection

The siRNA oligonucleotides against ADAR1 (Silencer Select s1007, ThermoFisher Scientific) and a negative control (Silencer Select Negative Control #1) were used. The oligonucleotides were transfected via the Lipofectamine RNAiMAX Transfection Reagent (#13778030, ThermoFisher Scientific) in line with the manufacturer's instructions.

Bacterial strains and LPS extraction

Fusobacterium nucleatum (ATCC 25586) and *Fusobacterium varium* (ATCC 8501) were used in this study. These bacteria were cultured at 37 °C under anaerobic conditions on a modified GAM medium. The extraction of the lipopolysaccharide (LPS) was carried out with LPS Extraction Kit (#17141, Intron,

Korea) according to the manufacturer's instructions.

RNA extraction and cDNA synthesis

Fresh frozen surgical specimens were homogenized with a Shakeman homogenizer (Bio Medical Science, Tokyo, Japan). The total RNA from tissues and cell lines was isolated using the RNeasy Mini kits (QIAGEN, Germantown, MD, USA) according to the manufacturer's instructions. The cDNA was synthesized from 1.0 μg of the total RNA using a High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, Waltham, MA, USA).

RNA editing site-specific quantitative PCR (RESS q-PCR)

The degree of editing of AZIN1 RNA was analyzed using the RNA editing site-specific quantitative polymerase chain reaction (RESS q-PCR) method published previously [6,13]. In brief, specific primers for wild-type and edited AZIN1 sequences were designed. Based on the difference in the Ct values, the ratios between the edited and wild-type AZIN1 were calculated using formula $2^{-(Ct\ Edited\ -\ Ct\ Wild\text{-}type)}$. Primer sequences for the PCRs are shown in **Supplementary Table 2**.

Real-time q-PCR analyses for ADAR1 and IFN-β

The real-time q-PCR was performed for gene expression analysis using the StepOne Real Time PCR System and Power SYBR Green Master Mix (Life Technologies, Carlsbad, CA, USA), as previously described [14,15]. GAPDH was used as a normalization control. The relative expression of each mRNA was determined using the ΔΔCt method. Primer sequences are shown in **Supplementary Table 2**.

Enzyme-linked immunosorbent assay

IFN-β levels were detected in the conditioned medium using ELISA assays (#DIFNB0, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Western immunoblotting

Western immunoblotting experiments were performed as described previously [6]. Anti-ADAR1 (1:500 dilution; ab88574, Abcam) antibody was used to detect the target protein, and an anti-β-actin antibody (1:1000, #A5441, Sigma-Aldrich, St. Louis, MD, USA) was used as the loading control.

Statistical analysis

Results are shown as means ± standard deviation. JMP software (ver.14.3.0 SAS Institute Inc., Cary, NC, USA) and Prism 9 (GraphPad Software, San Diego, CA, USA) were used to perform statistical analyses. Differences between groups were estimated by Wilcoxon's rank sum test, Wilcoxon's signed-rank sum test, Steel's test, and the Steel–Dwass test. When multiple hypothesis testing was performed, the P-value for significance was adjusted by Dunnett's correction in each analysis. Spearman's rank correlation analysis estimated correlations of two group analyses. Receiver operating characteristic (ROC) curves were established to distinguish UC patients with and without CAN. ROC curves determined the optimal cutoff values of ADAR1 intensity levels in logistic regression analysis with Youden's index. All p values were 2-sided, and those less than 0.05 were considered statistically significant.

RESULTS

The type 1 IFN-related inflammation pathway is upregulated in the rectum of active UC, rectum of UC-CAN, and tumor site of UC-CAN patients.

UC is a continuous inflammatory process that extends from the rectum to the cecum. We first wanted to characterize the inflammatory signals in UC. Gene expression analysis using next-generation sequencers suggested that type 1 IFN-related inflammation pathways are activated in the rectal mucosa in the acute phase of UC (UC Acute-rectum). Intriguingly, type 1 IFN-related inflammation pathways persist in the rectal mucosa (UC CAN-rectum) and cancerous lesions (UC CAN-CAN) in CAN cases (**Fig. 1**).

Based on these results, we hypothesized that inflammatory signals from persistent type 1 IFN expression, which cannot be detected by endoscopy, may contribute to carcinogenesis. ADAR1 is induced by the inflammatory cytokine type 1 IFN [7] and can accelerate carcinogenesis via oncogenic RNA editing [6,16]. Therefore, we investigated the involvement of RNA editing in CAN and its potential as a predictive marker of carcinogenesis.

ADAR1 expression was increased in UC samples according to the degree of inflammation.

There are virtually no reports of ADAR1 expression in UC. First, immunohistochemical staining was performed to determine whether ADAR1 expression was increased in UC samples. In this study, we adopted Mayo Endoscopic Score (MES) to evaluate the levels of inflammation. MES0 describes a case in complete remission, while MES1 to MES2 depict increasing inflammation (**Fig. 2A**).

We analyzed whether the expression of ADAR1 in the rectum correlates with the degree of inflammation during the surveillance phase. In MES0, MES1, and MES2, rectal biopsy tissue was immunostained to reveal ADAR1 (**Fig. 2B**). ADAR1 expression in MES1 and MES2 cases was higher than

that in MES0 cases ($p < 0.001$; **Fig. 2C**). This data indicates that ADAR1 expression in the rectum reflects the degree of inflammation in the colon in UC patients.

In UC, the degree of inflammation is said to be positively correlated with carcinogenesis [1]. Patients with MES1 and MES2 are at high risk for carcinogenesis and should be subject to surveillance by colonoscopy. However, it remains unclear how to extract cases that develop CAN, even in MES0.

ADAR1 expression may be a predictive marker for carcinogenesis in UC cases with MES0.

Carcinogenesis in MES0 cases requires special attention as detection is difficult when the inflammation seems to have subsided. Biomarkers are expected to predict such carcinogenesis, which cannot be predicted by MES classification based on endoscopic findings. Therefore, we formulated the following hypothesis. Even in MES0 cases, carcinogenesis may be promoted in patients with high ADAR1 levels that cannot be detected by endoscopy. If ADAR1 expression in the rectum can predict the degree of inflammation in the whole colon, immunostaining of ADAR1 only in the rectum may predict the development of CAN. We thus performed immunostaining for ADAR1 in biopsied tissue from MES0 cases.

We analyzed the expression of ADAR1 in the whole colon of MES0 cases. First, each CAN and non-CAN case was compared preliminarily. Interestingly, ADAR1 expression was upregulated in the entire colon of CAN cases, while it was down-regulated in that of non-CAN MES0 cases (**Fig. 2D**), suggesting that the prolonged inflammation proved by ADAR1 was not detectable by endoscopic findings. The high expression of ADAR1 may maintain an environment conducive to carcinogenesis, leading to field cancerization.

Next, we performed ADAR1 immunostaining in many MES0 cases to clarify the relationship

between ADAR1 expression and carcinogenesis. We compared the expression of ADAR1 in 14 CAN cases and seven non-CAN cases. In CAN cases, ADAR1 expression remained significantly higher in the whole colon, including the tumor site, than that in non-CAN cases (**Fig. 2E**). These results support our hypothesis that UC cases with a sustained environment of high ADAR1 expression induced by mild inflammation without endoscopic findings are more likely to develop CAN.

Even in MES0 cases, patients with persistently high ADAR1 expression may be at increased risk for carcinogenesis.

Based on the previous studies, we analyzed ADAR1 expression levels in the normal rectal mucosa, rectal mucosa in the acute phase (UC Acute-rectum), rectal mucosa of MES0 noncarcinomatous cases (UC Remission-rectum), rectal mucosa of MES0 CAN cases (UC CAN-rectum), and tumoral areas of MES0 CAN cases (UC CAN-CAN) (**Fig. 3A**).

ADAR1 in the rectum was elevated during active UC and decreased when inflammation subsided to MES0. In contrast, in CAN cases, ADAR1 expression remained high both in the rectal mucosa and in the tumoral area, even at MES0 ($p < 0.001$; **Fig. 3B**). These results indicate that in MES0 patients, the presence or absence of CAN may be predicted simply by assessing the level of ADAR1 expression in the rectal mucosa. Further analysis showed that ADAR1 expression in the rectum predicted the development of CAN in MES0 cases, with an area under the curve (AUC) of 0.93 ($p < 0.0001$; **Fig. 3C**).

ADAR1 is a better predictive marker for carcinogenesis in UC than p53 or β-catenin.

We compared the expression patterns of ADAR1 with the existing markers, p53 or β-catenin, to show

the advantage of ADAR1 in predicting the development of CAN because the mutations of p53 and accumulation of β-catenin are said to occur early in carcinogenesis in UC (**Fig. 3D**) [17-19].

In CAN cases, p53 was highly positive only in the tumoral area and almost negative in the noncancerous rectal mucosa, suggesting that the p53 mutation occurs only in the tumoral area and not in the noncancerous rectal mucosa. p53 was not considered a predictive marker for carcinogenesis by rectal mucosal biopsy. On the other hand, in CAN cases, β-catenin was highly expressed in both cancerous lesions and noncancerous rectal mucosa. However, the positive rate of β-catenin in the noncancerous rectal mucosa of CAN cases was only about 60%. Therefore, the expression of β-catenin in the rectal mucosa seemed to be underpowered as a predictive marker for carcinogenesis. The positive rate of ADAR1 expression in the rectal mucosa of CAN cases was nearly 100%. In non-CAN cases, the positive rate of ADAR1 expression in rectal mucosa was lower than 40% ($p < 0.05$), suggesting that ADAR1 expression analysis by rectal mucosa biopsy is superior to p53 ($p < 0.001$) and β-catenin ($p < 0.05$) as a predictive marker for CAN (**Fig. 3E**).

ADAR1 expression in the rectum can be examined to predict cases at high risk for CAN.

Based on these results, we expected that ADAR1 expression in rectal tissues could be applied to a novel biomarker in the surveillance protocol for patients in the remission period of UC.

Patients with UC do not require a total colonoscopy at the initial examination. ADAR1 expression may serve to identify patients requiring total colonoscopy using the following protocol. First, rectoscopy is used to screen patients with an MES greater than zero. Since these patients are at high risk for carcinogenesis, a total colonoscopy is required. If MES0 is detected by rectoscopy, immunostaining for ADAR1 is performed using biopsy material from the rectum. If ADAR1 expression is

high, the patient is at high risk of CAN and is indicated for total colonoscopy. In the case of low ADAR1 expression, the risk of CAN is low, so regular UC follow-up would be sufficient (**Fig. 4**). This protocol could precisely select high-risk patients requiring surveillance for inflammation or CAN and eliminate UC cases with less inflammation and no CAN lesions. This novel biomarker could offer a new screening test with less invasiveness and extract patients requiring surveillance colonoscopy.

ADAR1 promotes carcinogenesis by inducing AZIN1 RNA editing in UC patients.

We previously reported that a high expression of ADAR1 and the resulting AZIN1 RNA editing may be involved in carcinogenesis by inducing stemness [6]. We confirmed whether this carcinogenesis-promoting mechanism is functional in UC.

When we analyzed the UC biopsy material, we found that increased ADAR1 expression was positively correlated with increased AZIN1 RNA editing levels ($p < 0.001$, $\rho = 0.679$; **Fig. 5A**). Considering that ADAR1 is highly expressed in CAN cases, AZIN1 RNA editing promoted by ADAR1 may play a facilitative role in carcinogenesis in UC.

ADAR1 expression in UC may be triggered by type 1 IFN secreted from the macrophage.

Although ADAR1 is known to be induced by type 1 IFN [7], its source is problematic. Since UC is an inflammatory bowel disease, inflammatory cell infiltration is naturally observed. Macrophages play a significant role in the pathogenesis of UC exacerbations and CAN [20-22]. Therefore, we focused on macrophages as a source of type 1 IFN. Type 1 IFN supplied by macrophages may act on the colonic mucosa, inducing ADAR1 expression and promoting AZIN1 RNA editing, which may result in CAN (**Fig. 5B**). We therefore performed immunostaining for macrophages in clinical specimens of UC.

Using Iba-1, a marker of macrophages, immunostaining of clinical specimens of UC revealed strong expression of Iba-1 in the acute phase of UC (**Fig. 5C**). Interestingly, macrophage aggregation was decreased in the rectal mucosa of UC patients without CAN (UC Remission-rectum) but was maintained in the rectal mucosa (UC CAN-rectum) and at the site of CAN (UC CAN-CAN) in UC patients with CAN ($p < 0.05$; **Fig. 5D**). Among some MES0 patients, macrophage conglomerations may be maintained even though the inflammation appears to have subsided. In such patients, macrophages continue to supply type 1 IFN to the colonic mucosa and thus maintain ADAR1 expression. This phenomenon would mean that RNA editing of AZIN1 would remain high, probably promoting carcinogenesis. The strong positive correlation between macrophage aggregation and ADAR1 expression in the colonic mucosa of UC patients supports this hypothesis ($p < 0.001$, $\rho = 0.58$; **Fig. 5E**).

Induction of ADAR1 expression in UC may be an effect of the microbiome.

Finally, we addressed why macrophage aggregation is maintained and ADAR1 expression is continually induced. In UC, *Fusobacterium* is increased in the colonic mucosa [23-25]. *Fusobacterium* promotes the development of CAN [26]. We hypothesized that the induction of ADAR1 expression in UC might be due to a facilitating influence from the microbiome. LPS secreted from *Fusobacterium* stimulates the secretion of type 1 IFN from macrophages via Toll-like receptor 4 (TLR4) [27]. Type 1 IFN will act on colonic mucosa and enhance the transcriptional activity of ADAR1, thereby enhancing ADAR1 expression and AZIN1 RNA editing. This phenomenon will induce stemness and immortalization of the colonic mucosa, which is pro-carcinogenic (**Fig. 6A**).

To prove our hypothesis, we performed the following *in vitro* analysis. LPS was extracted from *Fusobacterium nucleatum* and *Fusobacterium varium* and added to THP1, and the culture supernatant

was added to HT-29, CACO-2, and RKO (**Fig. 6B**). When LPS from *Fusobacterium nucleatum* or *Fusobacterium varium* was added to THP1, secretion of IFN-β from THP1 was markedly increased ($p < 0.001$ in RNA, $p < 0.001$ in protein; **Fig. 6C**). We added THP1 medium cultured with LPS of *Fusobacterium nucleatum* or *Fusobacterium varium* to HT-29, RKO, and CACO-2 cells. Consistent with our hypothesis, LPS of *Fusobacterium* induced ADAR1 and increased AZIN1 RNA editing ($p < 0.05$), which were interfered with by siADAR1 ($p < 0.05$, **Fig. 6D-F**). These results indicate that *Fusobacterium spp.* LPS promotes ADAR1 expression and AZIN1 RNA editing in colon mucosal cells.

Not all causes of inflammation in UC are induced by the microbiome. However, it has been reported that *Fusobacterium spp.* proliferate in UC [23-25]. This study revealed that IFN stimulation from *Fusobacterium spp.* may promote RNA editing and contribute to carcinogenesis in patients with UC. RNA editing in UC could provide helpful information for surveillance and carcinogenesis prevention in the future.

DISCUSSION

Patients with UC can develop CAN. Surveillance guidelines in the United Kingdom are as follows [1]. All patients with UC should undergo colonoscopy to confirm the extent of disease eight years after disease onset. Early repeat surveillance colonoscopy is recommended for patients with primary sclerosing cholangitis because of their increased risk of CAN. However, for low risk (no active endoscopic or histological inflammation or left-sided colitis), intermediate-risk (mildly active endoscopic or histological inflammation), and high-risk patients (moderate / severely active endoscopic or histological inflammation), the next surveillance colonoscopy should be performed in five, three, and one years, respectively [1,28]. Colonoscopically targeted biopsies are recommended over random colonic biopsies. If

taking random biopsies, 2–4 biopsies should be taken every 10 cm [1,29,30].

Colonoscopic surveillance aims to detect CAN at an early, treatable stage. Rubin et al. reported that 33 to 64 biopsies are required to confirm CAN with 95% confidence [31]. On the other hand, Lynch et al. analyzed 12 papers reporting on surveillance in detail and noted that of 92 CAN detected in 1916 cases, only 11 were detected by actual surveillance colonoscopy [32]. Conventional random biopsy surveillance programs are inefficient and unsatisfactory for CAN detection.

There have been attempts to investigate molecular biological markers specific to CAN for more efficient surveillance. Aneuploidy, p53, loss of heterozygosity (LOH), and cell cycle genes have been studied as predictive markers of carcinogenesis. Especially, mutations in p53 occur early in the development of CAN [17,18], but late in developing sporadic CRC. p53 immunohistochemistry helps differentiate CAN from inflammatory regenerative atypical ducts, and intense staining is reported to be suggestive of CAN [33,34]. However, p53 gene mutations are early events in only 50% of patients with CAN [35]. Because of this lower sensitivity, p53 is unsuitable as a molecular biological marker.

Blind surveillance colonoscopy and existing molecular markers are inadequate for predicting carcinogenesis. Therefore, we decided to apply our knowledge of RNA editing, which we have developed through analysis of sporadic CRC, to UC. Our analysis of RNA editing in UC yielded the following key findings. First, the RNA-editing enzyme ADAR1 was highly expressed in UC, depending on the degree of inflammation. In particular, it became clear that even patients thought to have MES0 on endoscopic examination may have high expression of ADAR1 and prolonged inflammation at the molecular level. These results indicate that endoscopic observation alone cannot accurately assess the degree of inflammation. Second, even in UC cases classified as MES0, the high ADAR1 expression group is at high risk for CAN and seems to require surveillance by total colonoscopy as in the high

inflammatory groups, such as MES1 or 2. On the other hand, the low ADAR1 expression group is at low risk for CAN and does not require frequent colonoscopies or random biopsies. In particular, if only ADAR1 expression in the rectum is examined by immunostaining, it is possible to evaluate these risks without endoscopy. This approach is revolutionary because rectal biopsy can be performed under rectoscopy by a family physician. Third, the high expression of ADAR1 in UC may be triggered by type 1 IFN stimulation from UC-specific microbiomes, such as *Fusobacterium spp*. Although increases in *Fusobacterium nucleatum* and *Fusobacterium varium* in UC have been reported, its effects have remained unexplored. In this study, we show for the first time that the induction of AZIN1 RNA editing by ADAR1, whose expression is promoted by *Fusobacterium*, may induce carcinogenesis in UC.

A limitation of our present study is its retrospective design. We plan to initiate a prospective trial of a surveillance program based on risk classification by rectal ADAR1 expression in new UC patients. Lymphocytes, including T cells, are also essential components as a vital source of IFN production in UC. We want to continue our analysis, including T cells. Since we could not experiment with normal colon cells, we followed previous literature and used CACO-2 as the model [36].

In conclusion, ADAR1 expression in the rectum of UC patients can be used to determine the risk of CAN and identify those requiring colonoscopy. This technology will free UC patients from unnecessary colonoscopy and reduce their physical burden. In addition, the possibility that RNA editing is involved in carcinogenesis in UC has been suggested. This technology may facilitate the prevention and treatment of CAN in UC.

REFERENCES

1. Lamb CA, Kennedy NA, Raine T*, et al.* British society of gastroenterology consensus guidelines on the management of inflammatory bowel disease in adults. *Gut* 2019;**68**:s1-s106.

2. Ungaro R, Mehandru S, Allen PB, Peyrin-Biroulet L, Colombel JF. Ulcerative colitis. *Lancet* 2017;**389**:1756-70.

3. Jess T, Loftus EV, Jr., Velayos FS*, et al.* Risk of intestinal cancer in inflammatory bowel disease: A population-based study from olmsted county, minnesota. *Gastroenterology* 2006;**130**:1039-46.

4. Lennard-Jones JE, Morson BC, Ritchie JK, Williams CB. Cancer surveillance in ulcerative colitis. Experience over 15 years. *Lancet* 1983;**2**:149-52.

5. Schroeder KW, Tremaine WJ, Ilstrup DM. Coated oral 5-aminosalicylic acid therapy for mildly to moderately active ulcerative colitis. A randomized study. *The New England journal of medicine* 1987;**317**:1625-9.

6. Shigeyasu K, Okugawa Y, Toden S*, et al.* Azin1 rna editing confers cancer stemness and enhances oncogenic potential in colorectal cancer. *JCI insight* 2018;**3**.

7. Sun T, Yu Y, Wu X*, et al.* Decoupling expression and editing preferences of adar1 p150 and p110 isoforms. *Proceedings of the National Academy of Sciences of the United States of America* 2021;**118**.

8. Schneider CA, Rasband WS, Eliceiri KW. Nih image to imagej: 25 years of image analysis. *Nature methods* 2012;**9**:671-5.

9. Claessen MM, Schipper ME, Oldenburg B*, et al.* Wnt-pathway activation in ibd-associated colorectal carcinogenesis: Potential biomarkers for colonic surveillance. *Cellular oncology : the official journal of the International Society for Cellular Oncology* 2010;**32**:303-10.

10. Kim D, Langmead B, Salzberg SL. Hisat: A fast spliced aligner with low memory requirements. *Nature methods* 2015;**12**:357-60.

11. Anders S, Pyl PT, Huber W. Htseq--a python framework to work with high-throughput sequencing data. *Bioinformatics* 2015;**31**:166-9.

12. Subramanian A, Tamayo P, Mootha VK*, et al.* Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences of the United States of America* 2005;**102**:15545-50.

13. Crews LA, Jiang Q, Zipeto MA*, et al.* An rna editing fingerprint of cancer stem cell reprogramming. *Journal of translational medicine* 2015;**13**:52.

14. Okugawa Y, Toiyama Y, Hur K*, et al.* Metastasis-associated long non-coding rna drives gastric cancer development and promotes peritoneal metastasis. *Carcinogenesis* 2014;**35**:2731-9.

15. Komatsu Y, Shigeyasu K, Yano S*, et al.* Rna editing facilitates the enhanced production of neoantigens during the simultaneous administration of oxaliplatin and radiotherapy in colorectal cancer. *Scientific reports* 2022;**12**:13540.

16. Chen L, Li Y, Lin CH*, et al.* Recoding rna editing of azin1 predisposes to hepatocellular carcinoma. *Nat Med* 2013;**19**:209-16.

17. Brentnall TA, Crispin DA, Rabinovitch PS*, et al.* Mutations in the p53 gene: An early marker of neoplastic progression in ulcerative colitis. *Gastroenterology* 1994;**107**:369-78.

18. Holzmann K, Klump B, Borchard F*, et al.* Comparative analysis of histology, DNA content, p53 and ki-ras mutations in colectomy specimens with long-standing ulcerative colitis. *International journal of cancer* 1998;**76**:1-6.

19. Laurent C, Svrcek M, Flejou JF*, et al.* Immunohistochemical expression of cdx2, beta-catenin, and tp53 in inflammatory bowel disease-associated colorectal cancer. *Inflammatory bowel diseases* 2011;**17**:232-40.

20. Lee SH, Kwon JE, Cho ML. Immunological pathogenesis of inflammatory bowel disease. *Intestinal research* 2018;**16**:26-42.

21. Yao D, Dong M, Dai C, Wu S. Inflammation and inflammatory cytokine contribute to the initiation and development of ulcerative colitis and its associated cancer. *Inflammatory bowel diseases* 2019;**25**:1595-602.

22. Na YR, Stakenborg M, Seok SH, Matteoli G. Macrophages in intestinal inflammation and resolution: A potential therapeutic target in ibd. *Nature reviews Gastroenterology & hepatology* 2019;**16**:531-43.

23. Ohkusa T, Sato N, Ogihara T*, et al.* Fusobacterium varium localized in the colonic mucosa of patients with ulcerative colitis stimulates species-specific antibody. *Journal of gastroenterology and hepatology* 2002;**17**:849-53.

24. Yoshimatsu Y, Mikami Y, Kanai T. Bacteriotherapy for inflammatory bowel disease. *Inflammation and regeneration* 2021;**41**:3.

25. Su W, Chen Y, Cao P*, et al.* Fusobacterium nucleatum promotes the development of ulcerative colitis by inducing the autophagic cell death of intestinal epithelial. *Frontiers in cellular and infection microbiology* 2020;**10**:594806.

26. Yu MR, Kim HJ, Park HR. Fusobacterium nucleatum accelerates the progression of colitis-associated colorectal cancer by promoting emt. *Cancers* 2020;**12**.

27. Kawasaki T, Kawai T. Toll-like receptor signaling pathways. *Frontiers in immunology* 2014;**5**:461.

28. Cairns SR, Scholefield JH, Steele RJ*, et al.* Guidelines for colorectal cancer screening and surveillance in moderate and high risk groups (update from 2002). *Gut* 2010;**59**:666-89.

29. Gasia MF, Ghosh S, Panaccione R*, et al.* Targeted biopsies identify larger proportions of patients with colonic neoplasia undergoing high-definition colonoscopy, dye chromoendoscopy, or electronic virtual chromoendoscopy. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association* 2016;**14**:704-12 e4.

30. Magro F, Langner C, Driessen A*, et al.* European consensus on the histopathology of inflammatory bowel disease. *Journal of Crohn's & colitis* 2013;**7**:827-51.

31. Rubin CE, Haggitt RC, Burmer GC*, et al.* DNA aneuploidy in colonic biopsies predicts future development of dysplasia in ulcerative colitis. *Gastroenterology* 1992;**103**:1611-20.

32. Lynch DA, Lobo AJ, Sobala GM, Dixon MF, Axon AT. Failure of colonoscopic surveillance in ulcerative colitis. *Gut* 1993;**34**:1075-80.

33. Wong NA, Mayer NJ, MacKell S, Gilmour HM, Harrison DJ. Immunohistochemical assessment of ki67 and p53 expression assists the diagnosis and grading of ulcerative colitis-related dysplasia. *Histopathology* 2000;**37**:108-14.

34. Kobayashi K, Tomita H, Shimizu M*, et al.* P53 expression as a diagnostic biomarker in ulcerative colitis-associated cancer. *International journal of molecular sciences* 2017;**18**.

35. Foersch S, Neurath MF. Colitis-associated neoplasia: Molecular basis and clinical translation. *Cellular and molecular life sciences : CMLS* 2014;**71**:3523-35.

36. Jia YP, Wang K, Zhang ZJ*, et al.* Tlr2/tlr4 activation induces tregs and suppresses intestinal inflammation caused by fusobacterium nucleatum in vivo. *PloS one* 2017;**12**:e0186179.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article.

ACKNOWLEDGMENTS

We thank Tae Yamanishi and Tomoko Sueishi for their assistance with performing the experiments.

FIGURE LEGENDS

Figure 1: Type 1 IFN-related inflammation pathway is upregulated in UC patients with CAN.

Gene expression analysis using next-generation sequencers suggested that type 1 IFN-related inflammation pathways are activated in the rectal mucosa during the acute phase of UC (UC Acute-rectum). Type 1 IFN-related inflammation pathways persist in the rectal mucosa (UC CAN-rectum) and cancerous lesion (UC CAN-CAN) in CAN cases. UC: ulcerative colitis. CAN: colitis-associated colorectal neoplasm. IFN: interferon.

Figure 2: ADAR1 expression may be a predictive marker for carcinogenesis in ulcerative colitis.

(A) MES0 is a case in complete remission. MES1 to MES2 cases show increasing inflammation. **(B)** In MES0, MES1, and MES2, rectal biopsy tissue was used for immunostaining of ADAR1. **(C)** ADAR1 expression in MES1 and MES2 cases was higher than that in MES0 cases ($p < 0.001$). **(D)** ADAR1 expression was up-regulated in the entire colon of CAN cases, while it was down-regulated in that of non-CAN MES0 cases. **(E)** In CAN cases, ADAR1 expression remained significantly higher in the whole colon, including the tumor site, than that in non-CAN cases. CAN: colitis-associated colorectal neoplasm. MES: Mayo endoscopic score. ***$p < 0.001$.

Figure 3: ADAR1 is a better predictive marker for carcinogenesis in UC than β-catenin or p53.

(A) ADAR1 expression levels in the normal rectal mucosa, rectal mucosa in the acute phase (UC Acute-rectum), rectal mucosa of MES0 noncarcinomatous cases (UC Remission-rectum), rectal mucosa of MES0 CAN cases (UC CAN-rectum), and tumoral areas of MES0 CAN cases (UC CAN-CAN) were analyzed. **(B)** ADAR1 was elevated in the rectum during active UC and decreased when inflammation subsided to MES0. In contrast, ADAR1 expression remained high both in the rectal mucosa and in the tumoral area in CAN cases, even at MES0. **(C)** ADAR1 expression in the rectum predicted the development of colorectal cancer with an AUC of 0.93 ($p < 0.0001$). **(D, E)** The expression pattern of ADAR1 was compared with existing markers, p53 or β-catenin. ADAR1 expression analysis by rectal mucosa biopsy is superior to p53 and β-catenin as a predictive marker for CAN. UC: ulcerative colitis. CAN: colitis-associated colorectal neoplasm. AUC: area under the curve. MES: Mayo Endoscopic Score. *$p < 0.05$. ***$p < 0.001$.

Figure 4: ADAR1 expression in the rectum can be examined to predict cases of high risk of CAN.

If MES0 is detected by rectoscopy, immunostaining for ADAR1 can be performed using biopsy material from the rectum to identify patients at high risk of carcinogenesis. If ADAR1 expression is high, the patient is at high risk of CAN and is indicated for total colonoscopy. In the case of low ADAR1 expression, the risk of CAN is low, so regular UC follow-up would be sufficient. UC: ulcerative colitis. CAN: colitis-associated colorectal neoplasm.

Figure 5: ADAR1 expression in UC may be triggered by type 1 IFN secreted from the macrophage.

(A) Increased ADAR1 expression was positively correlated with increased AZIN1 RNA editing levels ($p < 0.001$, $\rho = 0.679$). **(B)** Type 1 IFN supplied by macrophages may act on the colonic mucosa, inducing ADAR1 expression and promoting AZIN1 RNA editing, which may result in CAN. **(C)** Using Iba-1, a marker of macrophages, immunostaining of clinical specimens of UC revealed strong Iba-1 expression in the acute phase of UC. **(D)** Macrophage aggregation was decreased in the rectal mucosa of UC patients without CAN (UC Remission-rectum) but was maintained in the rectal mucosa (UC CAN-rectum) and at the site of CAN (UC CAN-CAN) in UC CAN patients. **(E)** A strong positive correlation between macrophage aggregation and ADAR1 expression was observed in the colonic mucosa of UC patients ($p < 0.001$, $\rho = 0.58$). UC: ulcerative colitis. IFN: interferon. CAN: colitis-associated colorectal neoplasm. *$p < 0.05$.

Figure 6: Induction of ADAR1 expression in UC may be an effect of the microbiome.

(A) The induction of ADAR1 expression in UC might be due to a facilitating influence from the microbiome. **(B)** The following *in vitro* analysis was performed. LPS was extracted from *Fusobacterium* and added to the macrophage cell line THP1; then THP1 culture supernatant containing type 1 IFN was added to colon cells, and then we measured ADAR1 expression and AZIN1 RNA editing. **(C)** When LPS from *Fusobacterium nucleatum* or *Fusobacterium varium* was added to THP1, the secretion of IFN-β from THP1 was markedly increased. **(D-F)** LPS of *Fusobacterium* induced ADAR1 and increased AZIN1 RNA editing ($p < 0.05$), which were interfered with by siADAR1. UC: ulcerative colitis. IFN: interferon. LPS: Lipopolysaccharide. *$p < 0.05$. ***$p < 0.001$.