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ORIGINAL ARTICLE

Aberrant expression of MYD88 via RNA-controlling CNOT4 and EXOSC3 in colonic mucosa impacts generation of colonic cancer

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Abstract

In 2020, the worldwide incidence and mortality of colorectal cancer (CRC) were third and second, respectively. As the 5-y survival rate is low when CRC is diagnosed at an advanced stage, a reliable method to predict CRC susceptibility is important for preventing the onset and development and improving the prognosis of CRC. Therefore, we focused on the normal colonic mucosa to investigate changes in gene expression that may induce subsequent genetic alterations that induce malignant transformation. Comprehensive gene expression profiling in the normal mucosa adjacent to colon cancer (CC) compared with tissue from non-colon cancer patients was performed. PCR arrays and qRT-PCR revealed that the expression of 5 genes involved in the immune response, including *MYD88*, was increased in the normal mucosa of CC patients. The expression levels of MYD88 were strikingly increased in precancerous normal mucosa specimens, which harbored no somatic mutations, as shown by immunohistochemistry. Microarray analysis identified 2 novel RNA-controlling molecules, *EXOSC3* and *CNOT4*, that were significantly upregulated in the normal mucosa of CC patients and were clearly visualized in the nuclei. Forced expression of *EXOSC3* and *CNOT4* in human colonic epithelial cells increased the expression of *IFNGR1*, *MYD88*, *NFκBIA*, and *STAT3* and activated ERK1/2 and JNK in 293T cells. Taken together, these results suggested that, in the inflamed mucosa, EXOSC3- and CNOT4-mediated RNA stabilization, including that of *MYD88*, may trigger the development of cancer and can serve as a potential predictive marker and innovative treatment to control cancer development.

KEYWORDS
CNOT4, colon cancer, EXOSC3, MYD88, preventive medicine, RNA control

Abbreviations: CC, colon cancer; CIMP, CpG island methylator phenotype; CIN, chromosomal instability; CNOT4, Ccr4-Not4; CRC, colorectal cancer; EXOSC3, exosome component 3 (Rrp40); FOBT, fecal occult blood test; IHC, immunohistochemistry; JNK, Jun-N-terminal kinase; MAPK, mitogen-activated protein kinase; MMR, mismatch repair; MSI, microsatellite instability; MYD88, myeloid differentiation factor 88; SSL, sessile serrated lesion; TLR, Toll-like receptor.

1 | INTRODUCTION

Colorectal cancer (CRC) is the second and third most common cancer diagnosed in females and males, respectively, with an estimated 1.94 million cases and 929 500 deaths in 2020 worldwide.[1] When CRC is diagnosed at an early stage, the 5-y relative survival rate is very high,[2] and early resection of CRC allows for almost 100% recovery of the patients.[3] Therefore, a reliable way to predict CRC candidates is critical for cancer prevention and the early detection, diagnosis, and appropriate treatment of CRC.

Both genetic and epigenetic alterations commonly occur in CRC and are the driving force of tumorigenesis.[4-6] CRC arises from 1 or a combination of 3 different mechanisms: chromosomal instability (CIN), the CIMP, and microsatellite instability (MSI).[7,8] The CIN pathway is also known as the adenoma-carcinoma sequence.[9] CRC is generally considered to develop from benign lesions through the adenoma-carcinoma sequence and another tumorigenic pathway of de novo carcinogenesis, suggesting the development of tumors from normal (or intact) colonic mucosa without the intervening step of an adenoma.[10] It is well established that germline mutations in MSI-related genes occur in Lynch syndrome and are closely associated with colon cancer predisposition. In addition to traditional MSI testing and IHC analysis of mismatch repair (MMR) proteins, the evaluation of MSI by next-generation sequencing has been used to verify the presence of Lynch syndrome pancancer.[11-13]

Chronic inflammation has been shown to promote colon carcinogenesis by inducing genetic mutations, inhibiting apoptosis, and stimulating angiogenesis and cell proliferation.[14] Inflammation is an adaptive response that is triggered by a variety of abnormal conditions, including infection of the microbiome and tissue injury, as well as more subtle changes in tissue homeostasis.[15-17] Microbial infection or tissue injury triggers inflammation by activating the TLR/adaptor molecule myeloid differentiation factor 88 (MYD88) signaling pathway. MYD88 is a TLR adapter molecule that is important for the activation of downstream NF-κB and MAPK pathways,[18] contributing to carcinogenesis and prognosis in various cancers, including skin, liver, pancreatic, breast, lung, gastric, and colon cancer.[19-25] High expression of MYD88 was frequently detected in CRC with liver metastasis and significantly related to poor prognosis.[26] Experimentally, mice lacking MYD88 formed fewer tumors compared with wild-type mice with azoxymethane (AOM)-induced intestinal tumorigenesis. In APC^{MIN} mice, MYD88-dependent signaling was required for the progression of tumors derived from epithelial cells.[27] The role of MYD88 in controlling mucosal homeostasis depends on the effect of the transcription factor NF-κB on enterocyte survival, which is implicated in the inflammation associated with cancer.[25] Recently, *MYD88* gene mutations have been reported in hematological malignancies,[28,29] and increased protein expression of MYD88 with no somatic mutation was observed in gastric cancer.[30]

Multiple molecular events that occur in CRC have been thoroughly investigated by colon cancer cells with paired normal mucosa. Conversely, increasing evidence has indicated that clonal expansion of phenotypically normal or noncancerous tissues is biologically significant in cancer development.[31,32] Therefore, attention should be given to the molecular events occurring in the normal colonic mucosa to prevent CRC development. Therefore, we performed comprehensive molecular analyses using histologically normal colonic mucosa adjacent to colon cancer as a feasible precancerous lesion and compared these tissues to those without colon cancer. We identified 2 kinds of RNA-controlling components, EXOSC3 and CNOT4, that were markedly increased in the normal colonic mucosa of colon cancer patients with enhanced MYD88. Forced expression of EXOSC3 and CNOT4 increased the expression levels of *IFNGR1*, *MYD88*, *NFκBIA*, and *STAT3* in human colonic epithelial cells, leading to activation of the MAPK and JNK pathways. Therefore, disturbances in comprehensive RNA control in the normal colonic mucosa might allow for the onset of colon cancer, and EXOSC3 and CNOT4 would be potential predictive markers of colon cancer and contribute to the onset by stabilizing RNAs including *MYD88*.

2 | MATERIALS AND METHODS

2.1 | Patients and human tissues

Here, 17 CC patients and 15 non-colon cancer (NCC) patients who were diagnosed between 2008 and 2014 at the Department of Cancer Pathology, Faculty of Medicine, Hokkaido University, and Sapporo Tokushukai Hospital (Table 1) were included in the study. Among them, 2 representative cases were selected for PCR array and microarray analyses, which had the best quality of extracted RNAs and less infiltration of inflammatory cells in the normal mucosa. The 2 CC patients (CC11 and CC14) had sigmoid colon cancer with tumor sizes of 5.0 cm or less, and normal colonic mucosa 2.0 cm on the anal side from the tumor was used for the analyses. The 2 NCC patients (NCC1 and NCC2) had sigma elongatum or diverticulitis of the sigmoid colon. In each case, normal colonic mucosa without malignancy or inflammation, as observed microscopically, was used. All 17 CC and 15 NCC samples were used for immunohistochemical analysis of MYD88, EXOSC3, and CNOT4. All ethical issues related to human pathological specimens were discussed and approved by the Ethics Committee of Hokkaido University Graduate School of Medicine (Number 15-0035).

2.2 | Cell culture

Human colonic epithelial cells (HCoEpiCs) were purchased from ScienCell Research Laboratories and cultured in colonic epithelial cell medium (CoEpiCM, ScienCell Research Laboratories). Human embryonal kidney 293T cells were cultured in DMEM containing 10% FBS. The cells were cultured at 37°C in 5% CO_2.

TABLE 1 (a) Colon cancer (CC) patients. (b) Non-colon cancer (NCC) cases

(a)

Case no.	Age/sex	Macroscopic classification	Size	Depth of invasion	Tissue type	Disease stage
CC1	77M	2	5.0 × 3.5	SS	tub2	II
CC2	79M	2	5.0 × 3.5	SS	tub2>tub1	IIIa
CC3	71F	2	3.0 × 2.2	SS	tub2	II
CC4	81M	2	1.8 × 1.8	SS	tub2	II
CC5	68F	2	2.3 × 3.8	SS	tub2	IIIa
CC6	79F	2	2.8 × 2.8	MP	tub1	II
CC7	57F	2	2.5 × 3.5	SS	tub1>muc	IIIa
CC8	63F	2	1.8 × 1.8	MP	tub1>>tub2	IIIa
CC9	81M	2	5.0 × 4.5	SE	tub1>muc	II
CC10	54F	2	3.8 × 2.2	SS	tub2	IV
CC11	81M	2	5.0 × 3.0	SS	tub2>>tub1	II
CC12	95F	2	4.0 × 1.5	SE	tub2	IIIa
CC13	76M	2	2.6 × 2.6	SS	tub1>tub2	II
CC14	71M	2	5.0 × 4.0	SS	tub2	IIIa
CC15	83F	1	3.3 × 3.8	SS	tub2	II
CC16	79F	2	4.0 × 4.0	SS	tub2>tub1	II
CC17	64M	2	5.0 × 4.0	SE	tub1>tub2	IIIa

(b)

Case no.	Age/sex	Diagnosis
NCC1	64M	Megacolon
NCC2	87M	Colonic diverticulosis
NCC3	69M	Normal colonic mucosa
NCC4	76F	Colonic diverticulosis
NCC5	54M	Traumatic perforation
NCC6	57F	Compressive ulcer perforation
NCC7	77F	Colonic diverticulosis
NCC8	84F	Sigmoid colon perforation
NCC9	63M	Colonic diverticulosis
NCC10	88M	Colonic diverticulosis
NCC11	45F	Colonic diverticulosis
NCC12	57F	Compressive ulcer perforation
NCC13	67M	Colonic diverticulosis
NCC14	79M	Colonic diverticulosis
NCC15	30F	Sigmoid colon perforation

2.3 | RNA extraction from FFPE samples and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from formalin-fixed paraffin-embedded (FFPE) tissue sections of normal colonic mucosa from CC patients (CC11 and CC14) or NCC patients (NCC1 and NCC2) using the RNeasy FFPE Kit (Qiagen). All samples were cut at a thickness of 10 μm, attached to a glass slide, and trimmed only the normal mucosa area. cDNA was synthesized using a SuperScript VILO cDNA Synthesis Kit (Invitrogen), and qRT-PCR was performed using StepOnePlus™ instrument (Applied Biosystems, Thermo Fisher Scientific Inc.) as described previously.[33] Gene expression was determined using

Power SYBR Green PCR Master Mix (Thermo Fisher Scientific Inc). The primer sequences are listed in Table S1. Cycle threshold (C_T) values were determined using StepOne Software v.2.2.2 (Applied Biosystems, Thermo Fisher Scientific Inc.). The expression of total RNA relative to that of the control samples was normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and evaluated using the comparative C_T method.

2.4 | PCR array

Total RNA was extracted from the normal colonic mucosa of CC (CC11 and CC14) or NCC (NCC1 and NCC2) patients as described above, and PCR array analysis was performed using RT^2 Profiler™ PCR Array Human Innate & Adaptive Immune Responses (Qiagen; Table S2) according to the manufacturer's instructions.

2.5 | Microarray analysis

Total RNA was extracted from the normal colonic mucosa of CC (CC11 and CC14) or NCC (NCC1 and NCC2) patients as described above, and microarray analysis was performed using SurePrint G3 Human Gene Expression 8 × 60K v2 (Agilent). Gene expression in the normal mucosa of CC patients was analyzed and compared with that of NCC patients. After identifying the differentially expressed genes, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID).

2.6 | Immunohistochemistry (IHC)

To immunostain MYD88, deparaffinized sections were exposed to EnVision FLEX Target Retrieval Solution High pH (50×) (Dako) for 20 min at 97°C for activation, and Autostainer Link 48 (Dako) was used. IHC was performed using rabbit polyclonal anti-MyD88 antibodies (1:50 dilution; HFL-296, Santa Cruz Biotechnology, Inc). The sections were sensitized using EnVision FLEX+ Rabbit (LINKER) (Dako) for 15 min.

For immunohistochemical analysis of CNOT4 and EXOSC3, anti-CNOT4 (ab110013, Abcam) and anti-EXOSC3 (ab154400, Abcam) antibodies were used, and IHC was performed according to the standard protocol. The staining intensity was visually scored as follows: − (negative), 1+ (weak), 2+ (moderate), and 3+ (strong). The immunostaining of endothelial cells was evaluated as an internal control.

2.7 | Direct DNA sequencing of the MYD88 L265P mutation

Genomic DNA was isolated from the FFPE colon cancer tissues from 17 CC patients using a QIAmap DNA FFPE Tissue kit (Qiagen) according to the manufacturer's protocol. The primer sequences are listed in Table S1 and resulted in a DNA amplicon of 176 base pairs. The reaction mixture consisted of 25 μL of 2× GoTaq® Green Master Mix (Promega) and each primer (10 μmol/L). The mixture was subjected to 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, and ended with a final extension at 72°C for 5 min. Amplified DNA fragments were analyzed by 2% agarose gel electrophoresis under ultraviolet light after being stained with ethidium bromide, purified using a MinElute® PCR Purification Kit (Qiagen), and sequenced by the Sanger method.

2.8 | Transfection of 293T cells with EXOSC3 and CNOT4

Total RNA was extracted from human embryonic kidney 293F cells (RNeasy Mini Kit, Qiagen), and cDNA was synthesized using SuperScript VILO (Invitrogen). The coding regions of human EXOSC3 and CNOT4 were amplified by PCR using primers [*Kpn*I-EXOSC3-forward: 5′-GGTACCATGGCCGAACCTGCGTCTGT-3′ (underline: *Kpn*I site); *Xba*I-EXOSC3-reverse: 5′-TCTAGATCAACTTTCTGCCAATCTGG-3′ (underline: *Xba*I site); *Kpn*I-CNOT4-forward: 5′-GGTACCATGTCTCGCAGTCCTGATGC-3′ (underline: *Kpn*I site); *Xba*I-CNOT4-reverse: 5′-TCTAGAGGCCACAGTAGTGTGGAGAC-3′ (underline: *Xba*I site)]. The PCR products were cloned into the pCR®-TOPO-TA® vector (Thermo Fisher Scientific Inc.), and subcloned into the p2×FLAG-CMV2 vector (Life Technologies), which was integrated with the Flag-tag coding gene according to the manufacturer's instructions. The p2×FLAG-CMV2 vectors containing the *EXOSC3* or *CNOT4* gene were transfected into HCoEpiCs and 293F cells using FuGENE® HD transfection reagent (Promega).

2.9 | Immunoblotting

293T cells with or without forced expression of EXOSC3 and CNOT4 were lysed with lysis buffer (10 mmol/L Tris-HCl [pH 7.4], 5 mmol/L EDTA, 150 mmol/L NaCl, 10% glycerol, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride [PMSF], and 1 mmol/L sodium orthovanadate [Na_3VO_4]) and a protease inhibitor mixture (complete [EDTA-free] protease inhibitor [Roche]) for 20 min on ice and centrifuged at 15,106 *g* for 15 min at 4°C. Supernatants were subjected to SDS-PAGE, and the separated proteins were transferred to a polyvinylidene difluoride membrane (Millipore). The membranes were incubated with primary antibodies at 4°C overnight and then incubated with horseradish peroxidase-labeled secondary antibodies. The signals were developed using ECL™ Western Blotting Detection Reagents (GE Healthcare) and visualized with an LAS 4000 mini system (GE Healthcare). The antibodies used in this experiment were as follows: anti-FLAG® M2-peroxidase (HRP) (Sigma), anti-phospho-p44/42 MAPK (T202/Y204) (D13.14.4E) XP™, anti-phospho-JNK, anti-phospho-STAT3 (Y705), anti-phospho-AKT

(S473) (D9E) XP™ (Cell Signaling Technologies), anti-MYD88 (Santa Cruz Biotechnology), and anti-actin (clone C4) (Millipore).

3 | RESULTS

3.1 | Inflammation-related genes, including *MYD88*, are increased in paracancerous normal colonic mucosa

To determine the molecular changes that occur in precancerous colonic mucosa, we focused on identifying gene expression profiles in the paracancerous normal mucosa of CC patients and compared them to those in the normal mucosa of NCC patients. The CC patients were confirmed not to have Lynch syndrome by family history and IHC analysis for mismatch repair (MMR) proteins such as MLH1, MSH2, PMS2, and MSH6 (data not shown; Table 1). In addition, only CC patients with tumor sizes of 5.0 cm or less were selected for analysis. As representative samples with best quality of extracted RNAs and less infiltration of inflammatory cells in the normal mucosa, 2 CC patients with sigmoid colon cancer (CC11 and CC14) were selected, and normal colonic mucosa 2.0 cm on the anal side away from the tumor (NC11 and NC14) was used for the molecular analysis to exclude unpredictable effects of passage obstacles (Figure 1A). The normal mucosa was evaluated microscopically by pathologists and determined to be normal lesions without any inflammation or tumor components. In comparison, normal colonic mucosa from non-colon cancer patients (NCC1 and NCC2) were used. NCC1 and NCC2 had megacolon and diverticulitis of the sigmoid colon, respectively, and normal colonic mucosa close to the surgically resected margin in each patient was used (N1 and N2, Figure 1B).

For gene expression analyses, we first examined the relationship between inflammation and carcinogenesis by evaluating gene expression linked to inflammation using RT^2 Profiler™ PCR Array Human Innate & Adaptive Immune Responses in 4 sample sets (Figure 1C; Table S2). In the normal mucosa of 2 CC patients (NC11 and NC14), 21 and 12 genes were upregulated more than 2-fold relative to those in the non-colon cancer patients NCC1 (N1; Figure 2A) and NCC2 (N2; Figure 2B), respectively. The latter 12 genes were commonly and highly expressed than those in N1 and N2 (Figure 2C).

To verify the PCR array results, qRT-PCR was performed for 12 commonly upregulated genes in the 2 CC cases (*DDX58*, *HLA-E*, *IFNGR1*, *IL18*, *IL1R1*, *IRF3*, *MAPK1*, *MAPK8*, *MYD88*, *NFKBIA*, *STAT1*, and *STAT3*) (Table S1). Among them, 5 genes (*IFNGR1*, *IL18*, *MYD88*, *NFKBIA*, and *STAT3*) were increased more than 2-fold in the normal colonic mucosa of 2 CC patients (NC11 and NC14) compared with those of NCC patients (N1 and N2) (Figure 2D). However, the other 7 genes were not verified by qRT-PCR (Figure S1). Particularly, *HLA-E* that had the highest expression in PCR array was investigated using another 8 N samples and 8 NC samples; the means ± SD in NC samples and N samples were 0.29 ± 0.14 and 0.59 ± 0.22, respectively, indicating lower expression in the NC samples (Figure S1B).

3.2 | MYD88 is increased in the normal colonic mucosa of colon cancer patients

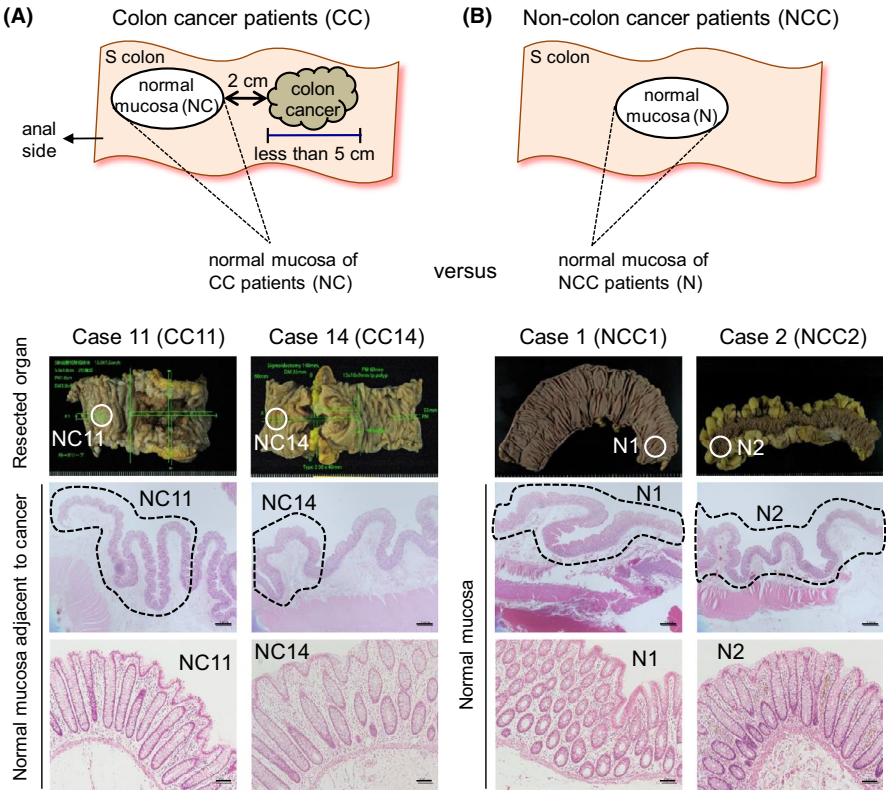
Because the adaptor protein MYD88 has been shown to contribute to carcinogenesis and is related to prognosis in many cancers, including skin, liver, pancreatic, breast, lung, gastric, and colon cancer,[19-25] we investigated the protein expression of MYD88 in paracancerous normal colonic mucosa. To determine MYD88 expression in clinical samples, immunohistochemistry (IHC) was performed on 17 CC patients and 15 NCC samples. The 17 CC cases were confirmed to harbor KRAS mutations such as G12V and G13D by Sanger sequencing, suggesting the onset through adenoma-carcinoma sequence. MYD88 was diffusely localized in the cytoplasm (Figure 3A) in paracancerous normal mucosa in all CC cases (1+: 8 cases [47%], 2+: 8 cases [47%], 3+: 1 case [5.9%], average score: 1.59). Notably, MYD88 seemed to be downregulated in the paired tumor lesions of approximately half of the cases (negative: 7 cases [41%], 1+: 5 cases [29%], 2+: 3 cases [18%], 3+: 2 cases [12%], average score: 1.00; Figure 3B,C). Moreover, in normal colonic mucosa without colon cancer, the expression levels were low (negative: 1 case [6.7%], 1+: 14 cases (93%), average score: 0.93; Figure 3B,C). Therefore, consistent with the gene expression data, MYD88 protein expression was higher in normal colonic mucosa in CC patients than in NCC patients (Figure 3C).

3.3 | Mutation analysis of MYD88 in colon cancer tissues

Recent studies have revealed the MYD88 L265P somatic mutation in hematological malignancies that induces constitutive activation of NF-κB signaling and tumorigenesis.[28,29] To examine the mutation status of MYD88 in colon cancer patients, we screened the L265P hot-spot mutation in 17 colon cancer samples by direct sequencing, but no somatic mutations were found in any samples (Figure 3D).

3.4 | Comprehensive gene expression profiling identified increases in RNA-controlling EXOSC3 and CNOT4 in paracancerous normal colonic mucosa

To comprehensively investigate the gene expression profile in the mucosa of colon cancer patients, microarray analysis was performed using normal colonic mucosa with or without colon cancer. Similar to the inflammation-related PCR array (Figures 1 and 2), 4 sample sets of normal colonic mucosa with or without colon cancer (N1 vs. NC11 or NC14, N2 *vs*. NC11 or NC14) were analyzed. Compared with those in the normal mucosa of NCC patients, 257, 98, and 30 genes were upregulated more than 2-fold, 2.5-fold, and 3-fold by the log2 ratio, respectively, in the paracancerous normal mucosa (Figure 4A). Among the 5 genes identified by PCR array and qRT-PCR (Figure 2D), reproducible increases were observed in *IFNGR1*, *IL18*, *MYD88*, and *STAT3* but not *NFKBIA*, as shown by



(C)

Comparison of normal mucosa	Colon cancer patient (CC)	Non-colon cancer patient (NCC)
Set 01	CC11 (NC11)	NCC1 (N1)
Set 02	CC14 (NC14)	NCC1 (N1)
Set 03	CC11 (NC11)	NCC2 (N2)
Set 04	CC14 (NC14)	NCC2 (N2)

FIGURE 1 Macroscopic and microscopic images of the normal colonic mucosa of colon cancer patients and non-colon cancer patients as controls. A, The normal colonic mucosa in of cancer patients. In sigmoid colon cancer patients with tumor sizes <5.0 cm, normal colonic mucosa 2.0 cm on the anal side of the tumor was used for the study (upper diagram). Macroscopic images of surgically resected tissues from colon cancer patients (CC11 and CC14) are displayed in the top row. The micrographs (NC11 and NC14) show normal colonic mucosa tissue 2.0 cm to the anal side of the tumor in each patient at low (middle) and high magnifications (bottom). Scale bar; 1.0 mm (middle) and 100 μm (bottom). B, The normal colonic mucosa of non-colon cancer patients (NCC1 and NCC2). NCC1 and NCC2 have sigma elongatum and diverticulitis of the sigmoid colon, respectively. Normal colonic mucosa adjacent to the surgically resected margin in each patient was used for this analysis (N1 and N2). Scale bar; 1.0 mm (middle) and 100 μm (bottom). C, Four sets of samples were used for PCR array, qRT-PCR, and microarray analyses. CC, colon cancer patient; N, normal mucosa in non-colon cancer patients; NC, normal mucosa in colon cancer patients; NCC, non-colon cancer patient

microarray analysis (data not shown). Among them, IFNGR-1 has been reported to play a role in tissue repair and cancer surveillance and, in the present study, immunohistochemical analysis demonstrated that IFNGR-1 was highly expressed in paracancerous normal mucosa (2+: 3 cases (17.6%), 3+: 14 cases (82.4%), average score: 2.82; Figure S2A,B). Interestingly, expression was decreased in the paired tumor lesions in 58.8% of the cases (negative: 10 cases [58.8%], 2+: 2 cases [11.8%], 3+: 5 cases [29.4%], average score: 1.12; Figure S2B), similar to MYD88 (Figure 3). The expression levels of NF-κB were increased in most normal mucosa with colon cancer, but the results varied depending on the case (Figure S3).

KEGG pathway analysis identified that 4 genes (*PI3K*, *PKC*, *Grb2*, and *β-catenin*) that have been reported to be broadly activated in various types of cancer cells were also upregulated in the paracancerous normal colonic mucosa (Figure 4B). Indeed, β-catenin was highly expressed in the normal mucosa of 17 CC patients (2+: 17 cases [100%]) in addition to the cancer lesions, and this gene was also highly expressed in the normal mucosa of 15 NCC cases (2+: 4 cases (26.7%), 3+: 11 cases (73.3%), average score: 2.73; Figure S4), indicating that expression occurred regardless of cancer status. Notably, RNA-controlling genes such as *EXOSC3* (*exosome component 3*, *Rrp40*) and *CNOT4* (*Ccr4-Not4*) were markedly increased in the paracancerous normal mucosa at

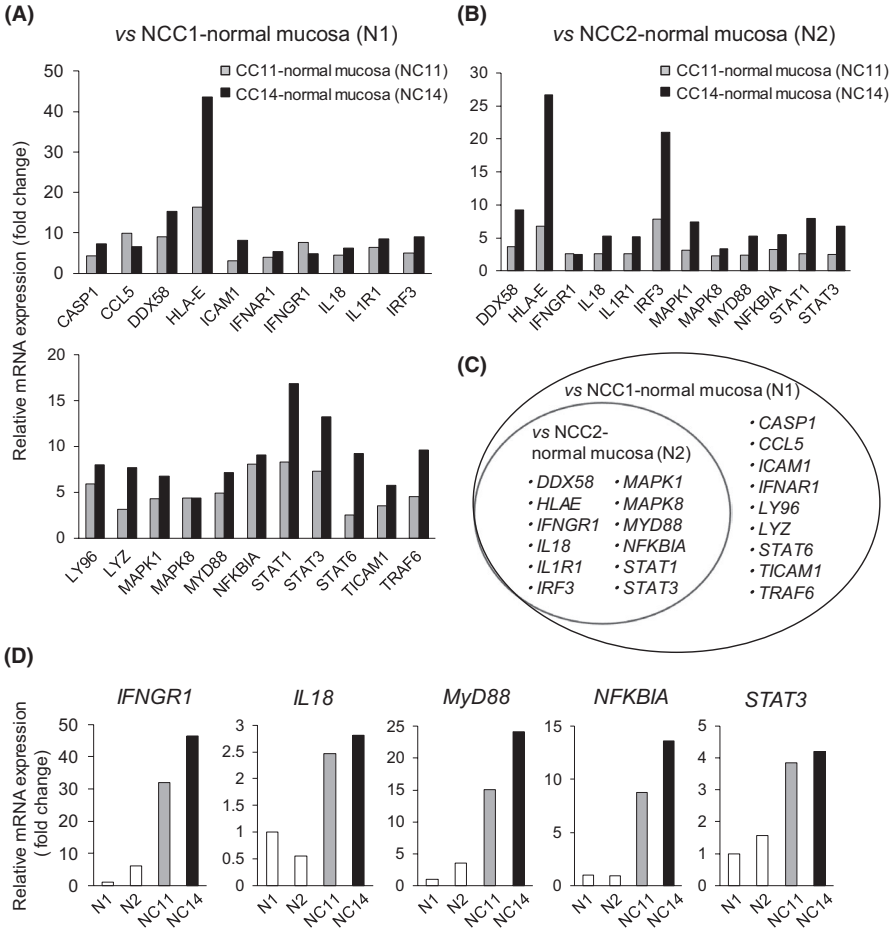


FIGURE 2 Upregulated genes in the paracancerous normal colonic mucosa of colon cancer patients, as determined by PCR array and qRT-PCR. A, B, In the PCR array, 21 and 12 genes were upregulated more than two-fold in the normal colonic mucosa of 2 CC patients (NC11 and NC14) compared with those of NCC1 (N1, A) and NCC2 (N2, B), respectively. C, Venn diagram displaying the upregulated genes in the normal colonic mucosa of 2 CC patients (NC11 and NC14) compared with NCC1 and NCC2. D, mRNA expression levels were determined by qRT-PCR. Among the 12 genes that were commonly upregulated in the PCR array, 5 genes (*IFNGR1*, *IL18*, *MyD88*, *NFKBIA*, and *STAT3*) were confirmed to be upregulated more than 2.0-fold in the normal mucosa of CC patients (NC11 and NC14) compared with that of NCC patients (N1 and N2) by qRT-PCR. The expression levels in the N1 (A and C) and the N2 (B) were set as 1.0 and the relative expression were shown

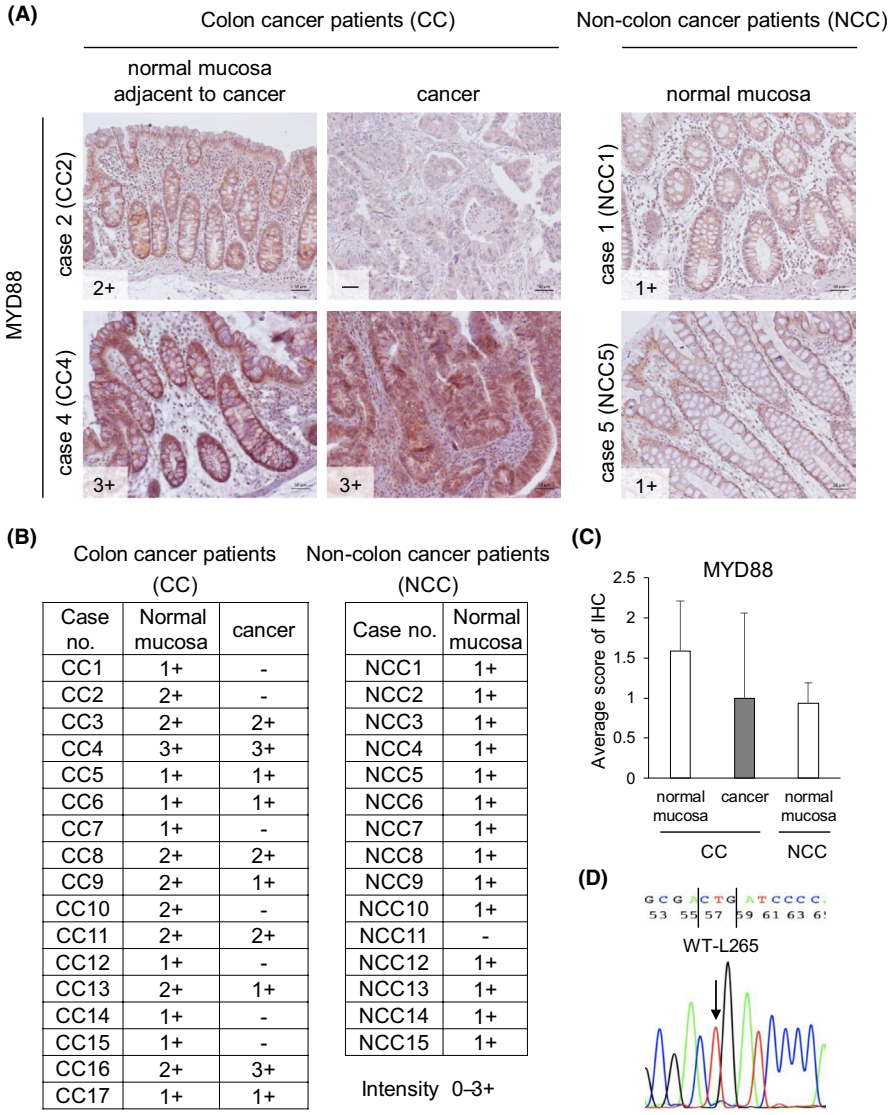
a log2 ratio >3 (Figure 4B). qRT-PCR analysis confirmed this enhancement (Figure 4C).

3.5 | EXOSC3 and CNOT4 are increased in paracancerous normal colonic mucosa

To verify the microarray analysis data, immunohistochemical analysis of EXOSC3 and CNOT4 was performed. Both EXOSC3 and CNOT4 were strongly localized to the nucleus (Figures 5A and 6A), particularly in nucleoli as dot-like structures in the proliferative zone (Figures 5B and 6B). EXOSC3 was highly expressed in the paracancerous normal mucosa of all CC cases (1+: 5 cases [29.4%], 2+: 12 cases [70.6%], average score: 1.71), whereas expression was rare in the mucosa of NCC cases (negative: 13 cases [86.7%], 1+: 2 cases [13.3%], average score: 0.13; Figure 5C,D). CNOT4 was also detected in the normal mucosa neighboring colon cancer (1+: 2 cases [11.8%], 2+: 9 cases [52.9%], 3+: 6 cases [35.3%], average score: 2.24), while in patients without colon cancer, expression was low (negative: 9 cases [60.0%], 1+: 5 cases [33.3%], 2+: 1 case [6.7%], average score: 0.47; Figure 6C,D). We also investigated the expression of EXOSC3, CNOT4 and MYD88 in 17 cases with SSL (Figure S5A), because a majority of colon cancer develops also through the serrated-neoplasia pathway in addition to the adenoma-carcinoma sequence. The expression of MYD88 and EXOSC3 was almost equal between SSL and the corresponding adjacent normal mucosa. In addition, the expression of MYD88 and EXOSC3 is likely to correlate with each other. Conversely, the expression levels of CNOT4 were low in normal mucosa in all cases (1+ or 0), whereas they were clearly enhanced in SSL, which is specific for the serrated pathway but not in the adenoma-carcinoma sequence. Therefore, CNOT4 might be dominant in controlling the expression levels of genes in the SSL (Figure S5B).

3.6 | Overexpression of EXOSC3 and CNOT4 activates the JNK and MAPK pathways in 293T cells

To clarify the causal relationship between the increased expression of EXOSC3 or CNOT4 and the development of colorectal cancer, we cloned cDNAs of EXOSC3 and CNOT4 and overexpressed them in human colonic epithelial cells (HCoEpiCs, ScienCell Research Laboratories; Figure 7A). Of the 5 genes identified by PCR array and qRT-PCR (Figure 2), *IFNGR1*, *MYD88*, *NFKB1A*, and *STAT* were increased by the forced expression of EXOSC3 and CNOT4, especially by EXOSC3 (Figure 7B). Similar results were observed in 293T cells, with slightly lower expression levels than those of HCoEpiC (Figure S6), suggesting that the HCoEpiC has a more well established machinery for expressing *IFNGR1*, *MYD88*, *NFKB1A*, and *STAT3*, which might contribute to the onset of colon cancer. To investigate cellular signaling, we used 293T cells because the transfection efficiency



Case no.	Normal mucosa	cancer
CC1	1+	-
CC2	2+	-
CC3	2+	2+
CC4	3+	3+
CC5	1+	1+
CC6	1+	1+
CC7	1+	-
CC8	2+	2+
CC9	2+	1+
CC10	2+	-
CC11	2+	2+
CC12	1+	-
CC13	2+	1+
CC14	1+	-
CC15	1+	-
CC16	2+	3+
CC17	1+	1+

Case no.	Normal mucosa
NCC1	1+
NCC2	1+
NCC3	1+
NCC4	1+
NCC5	1+
NCC6	1+
NCC7	1+
NCC8	1+
NCC9	1+
NCC10	1+
NCC11	-
NCC12	1+
NCC13	1+
NCC14	1+
NCC15	1+

Intensity 0–3+

FIGURE 3 Immunohistochemical staining of MYD88 in CC patients and NCC patients. A, Representative immunostaining images of MYD88 in the paracancerous normal colonic mucosa (left) and paired cancer lesions (middle) of CC patients and the normal colonic mucosa of NCC patients (right). B, Summary of immunohistochemical analysis of MYD88 in all cases (17 CC patients and 15 NCC patients) is shown. − (negative), 1+ (weak), 2+ (moderate), 3+ (strong). C, Average MYD88 IHC scores were calculated as the average of the total sum of intensity × case number and graphed with standard deviation. D, Direct sequencing of the 176 bp PCR-amplified MYD88 gene product was performed in tumor tissues from 17 CC patients. Representative sequence chromatograms including coding regions of the 265th amino acid are reported as hot spots were shown. Arrow indicates T in wild-type

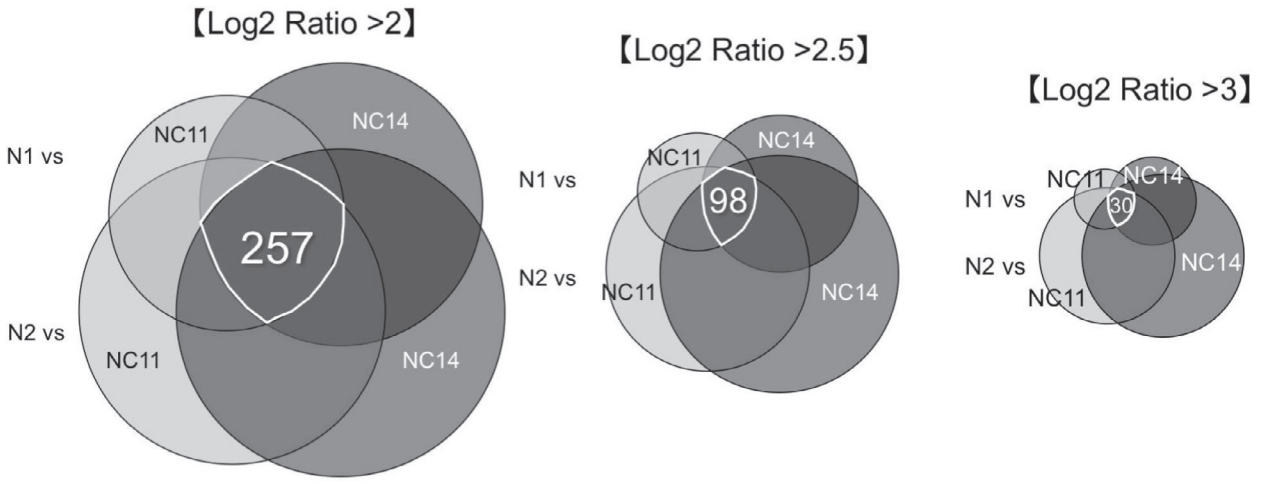
in HCoEpiCs was low and insufficient to detect the target factors. Immunoblotting revealed that the forced expression of EXOSC3 and CNOT4 increased the phosphorylation levels of MAPKs, especially phospho-JNK and phospho-ERK1/2 (Figure 7A,B). MYD88 expression was increased by the overexpression of EXOSC3 and CNOT4, especially by EXOSC3 (Figure 7C,D).

4 | DISCUSSION

Colorectal cancer (CRC) is one of most common cancers worldwide and is curable when the disease is discovered at an early stage. For the early detection and diagnosis of CRC, the FOBT and blood tests for tumor markers are commonly available for screening, but the sensitivity is low. Colonoscopy is a more accurate and reliable method for detecting CRC at the early phase, however it the method inadequate for screening because it is more invasive and expensive than blood tests.[2] In addition to early detection of CRC, the establishment of a prediction method is also important for preventing the onset of colon cancer. Genetic and epigenetic changes play critical roles in the onset and development of CRC, and several papers have reported that it may be valuable to use quantitative objective measures of epigenetic changes in normal tissues to detect CRC or precancerous lesions.[34-39] Although comparisons of DNA methylation

FIGURE 4 Genes that were upregulated in the paracancerous normal colonic mucosa of colon cancer patients, as determined by microarray analysis. A, Venn diagrams displaying the upregulated genes in the normal colonic mucosa of CC11 and CC14 compared with NCC1 and NCC2. In the paracancerous normal mucosa, the expression levels of 257, 98, and 30 genes were increased more than 2-fold, 2.5-fold, and 3-fold in the log2 ratio, respectively. The size of the circles on the Venn diagrams match the number of genes. B, Using the data obtained from the microarray analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses of the upregulated genes were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID). The upregulated genes with log2 ratios >2, >2,5, and >3 are displayed. C, qRT-PCR was performed to analyze *EXOSC3 var2* and *CNOT4* mRNAs

(A)



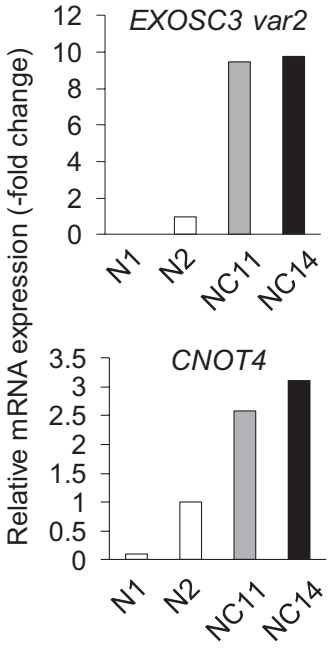
(B)

Log2 Ratio >2	Fc gamma R-mediated phagocytosis	PI3K	PKC			FcgRIIA	Src	PAP			
	Non-small-cell lung cancer	PI3K	PKC	Grb2		Cyclin D1					
	Wnt signaling pathway		PKC		β-catenin	FRP	Axin	Siah-1	Prickle		
	Glioma	PI3K	PKC	Grb2		ERK	E2F				
	B cell receptor signaling	PI3K	PKCb	Grb2		Lyn					
	Fc epsilon RI signaling	PI3K	PKC	Grb2		Lyn					
	Pathway in cancer	PI3K	PKC	Grb2	β-catenin	E2F	APC	APPL	FGFR	PPFR	PPARg
	Insulin signaling pathway	PI3K		Grb2		PKA	Rheb	LAR			
	Focal adhesion	PI3K	PKC	Grb2	β-catenin	MLCP	ECM				
	Colorectal cancer	PI3K			β-catenin	APPL					
	Prostate cancer	PI3K		Grb2	β-catenin	E2F					
	Endometrial cancer	PI3K		Grb2	β-catenin						

Log2 Ratio >2.5	Fc gamma R-mediated phagocytosis	PI3K	PKC			FcgRIIA
	Non-small-cell lung cancer	PI3K	PKC	Grb2		
	Glioma	PI3K	PKCb	Grb2		ERK
	B cell receptor signaling	PI3K	PKC	Grb2		
	Fc epsilon RI signaling	PI3K	PKC	Grb2		
	Focal adhesion	PI4K	PKC	Grb2		MLCP
	ErbB signaling pathway	PI3K	PKC			
	Natural killer cell-mediated cytotoxicity	PI3K	PKC	Grb2		

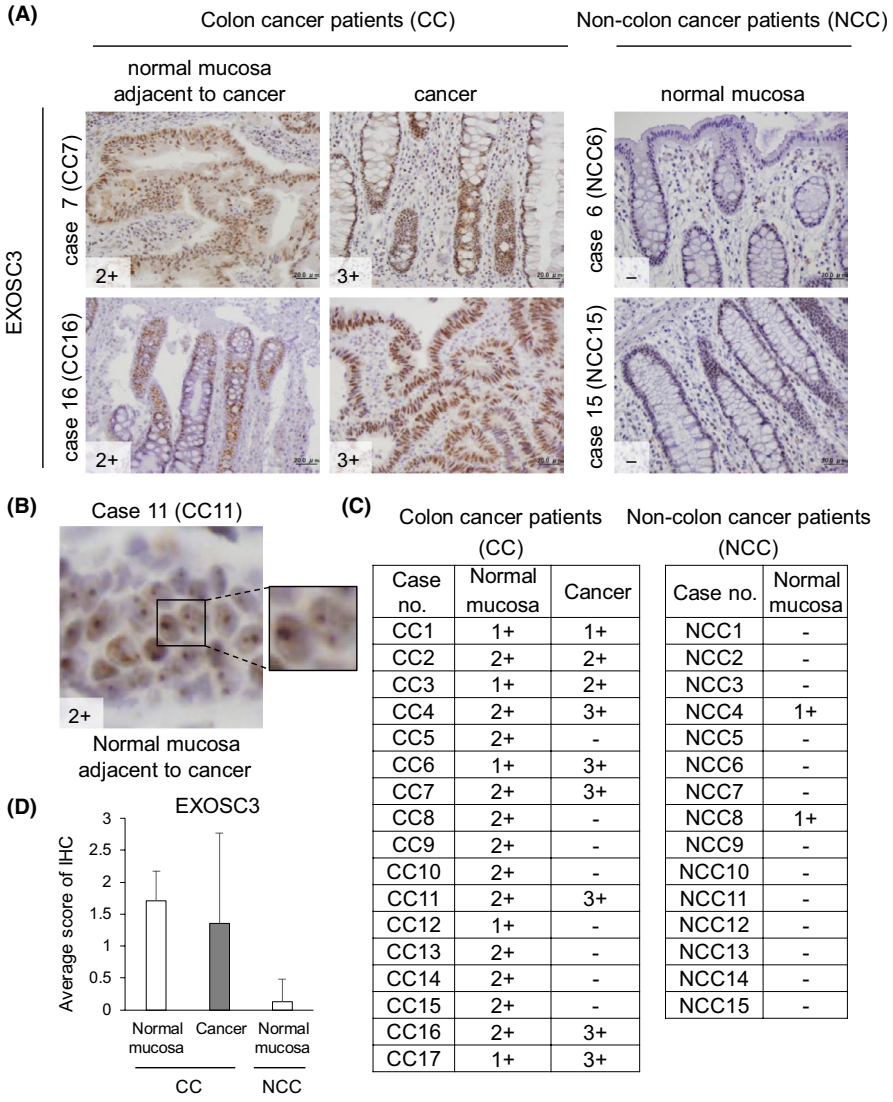
Log2 Ratio >3	RNA degradation					Rrp40	CNOT4

(C)



status in the normal colonic mucosa of patients with or without colonic cancer have been reported,[34] this method seems to be inadequate in the clinic.

Individual CRC susceptibility seems to differ in the background normal colonic mucosa under the influence of inflammation. The prediction of CRC susceptibility is valuable for the early detection



Case no.	Normal mucosa	Cancer
CC1	1+	1+
CC2	2+	2+
CC3	1+	2+
CC4	2+	3+
CC5	2+	-
CC6	1+	3+
CC7	2+	3+
CC8	2+	-
CC9	2+	-
CC10	2+	-
CC11	2+	3+
CC12	1+	-
CC13	2+	-
CC14	2+	-
CC15	2+	-
CC16	2+	3+
CC17	1+	3+

Case no.	Normal mucosa
NCC1	-
NCC2	-
NCC3	-
NCC4	1+
NCC5	-
NCC6	-
NCC7	-
NCC8	1+
NCC9	-
NCC10	-
NCC11	-
NCC12	-
NCC13	-
NCC14	-
NCC15	-

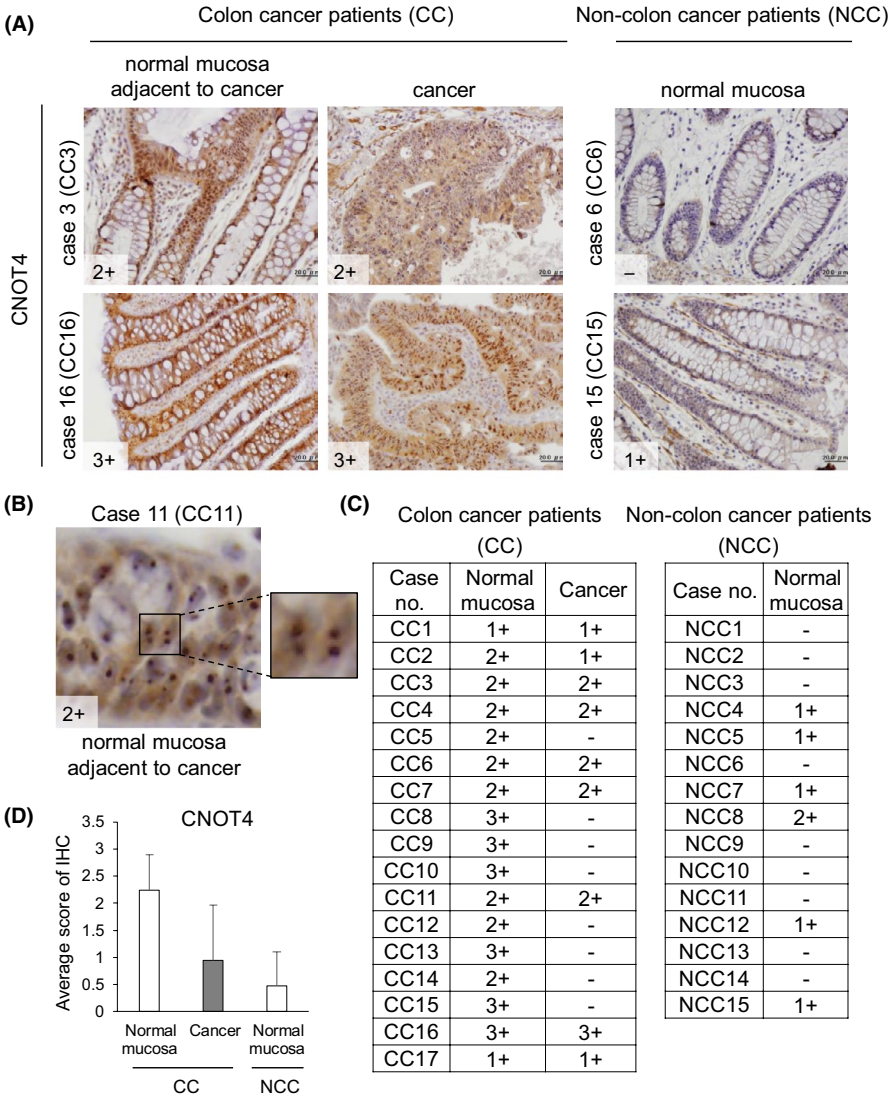
FIGURE 5 EXOSC3 was highly expressed in the paracancerous normal colonic mucosa. A, Immunohistochemical analysis of EXOSC3 was performed on samples from 17 CC patients and 15 NCC patients, and representative images of the paracancerous normal colonic mucosa (left) and paired cancer lesions (middle) of CC patients and the normal mucosa of NCC patients (right) are shown. B, Higher magnification image of EXOSC3 immunostaining in the normal mucosa of CC patients. C, Summary of immunohistochemical analysis of EXOSC3 in all cases is shown. – (negative), 1+ (weak), 2+ (moderate), 3+ (strong). D, The average scores of EXOSC3 were calculated as the average of the total sum of intensity × case number, and the results are graphed with standard deviation

and appropriate treatment of CRC at an early stage. In this study, to identify the changes in gene expression that occur in the normal mucosa before genetic and epigenetic changes, comprehensive gene expression profiling was performed in the precancerous normal mucosa. Among the 5 genes that were upregulated in paracancerous normal mucosa, *IFNGR1*, *IL18*, *MyD88*, *NFKBIA*, and *STAT3* (Figure 2), IL18 has been reported to suppress dysbiosis (abnormalities such as component changes in the intestinal flora), induce the production of IFN-γ in the surrounding interstitium, and exert antitumor effects such as tissue repair and tumor surveillance through IFNGR1.[40,41] Moreover, the expression levels of *MyD88* were increased 15-fold to 24-fold in paracancerous normal colonic mucosa compared with in the mucosa of NCC patients (Figure 2D), suggesting that the histologically diagnosed "normal" colonic mucosa may have already acquired several molecular changes, presumably due to inflammation-induced changes in the colonic microenvironment leading to the onset of colon cancer.

MYD88 has been shown to contribute to carcinogenesis, tumor development, and chemoresistance in various cancers. MYD88-related signaling influences both tumor-mediated promotion of extrinsic inflammation mediated by infiltrating hematopoietic cells and the intrinsically induced inflammation mediated by oncogene-induced cell transformation. Regardless of the cause, several studies have reported that MYD88 expression is decreased in cancerous tissues compared with paracancerous normal tissues and as cancer progresses.[26,30,42] Our study, IHC demonstrated similar phenomena in approximately half of the cases (Figure 3B). MYD88-mediated pathways were preventively involved in cancer promotion in several mouse carcinogenesis models.[43] Under chronic colitis conditions induced by DSS-induced mucosal damage, MYD88 plays a protective role against colon cancer.[43] Therefore, MYD88 seems to have divergent effects on the development of cancer.

Recently, somatic mutations in MYD88, such as L265P, have been reported in hematological malignancies,[28,29] and increased protein expression of MYD88 with no mutation was observed in gastric cancer.[30] In this study, we found no somatic mutations in MYD88 in the tumor lesions of all colon cancer patients (Figure 3D), indicating mutation-independent upregulation of MYD88.

Here, we identified 2 novel RNA-controlling molecules, EXOSC3 and CNOT4, that were upregulated in paracancerous



Case no.	Normal mucosa	Cancer
CC1	1+	1+
CC2	2+	1+
CC3	2+	2+
CC4	2+	2+
CC5	2+	-
CC6	2+	2+
CC7	2+	2+
CC8	3+	-
CC9	3+	-
CC10	3+	-
CC11	2+	2+
CC12	2+	-
CC13	3+	-
CC14	2+	-
CC15	3+	-
CC16	3+	3+
CC17	1+	1+

Case no.	Normal mucosa
NCC1	-
NCC2	-
NCC3	-
NCC4	1+
NCC5	1+
NCC6	-
NCC7	1+
NCC8	2+
NCC9	-
NCC10	-
NCC11	-
NCC12	1+
NCC13	-
NCC14	-
NCC15	1+

FIGURE 6 CNOT4 was significantly expressed in normal mucosa adjacent to colon cancer. A, Immunohistochemical analysis of CNOT4 was performed on samples from 17 CC patients and 15 NCC patients, and representative images of the paracancerous normal colonic mucosa (left) and paired tumor lesions (middle) of CC patients and the normal mucosa of NCC patients (right) are shown. B, Higher magnification image of CNOT4 immunostaining in the normal mucosa of CC patients. C, Summary of the immunohistochemical analysis of CNOT4 in all cases is shown. – (negative), 1+ (weak), 2+ (moderate), 3+ (strong). D, Average CNOT4 IHC scores were calculated as the average of the total sum of intensity × case number and graphed with standard deviation

normal colonic mucosa (Figures 4-6). EXOSC3 (exosome component 3 (Rrp40)) is one of the constituent elements of RNA exosomes, which are large complexes that are responsible for 3′–5′ exonuclease activity, and are involved in RNA degradation and posttranscriptional modification. EXOSC3 has S1 and KH RNA-binding domains.[44-46] EXOSC3 mutations have been reported to cause pontocerebellar hypoplasia and spinal motor neuron degeneration and shedding,[47,48] but no association with colon cancer has been reported therefore far. CNOT4 (Ccr4-Not4) is a component of the mRNA degrading enzyme complex Ccr4-Not and is involved not only in the degradation of mRNA but also in the regulation of overall mRNA metabolism from the transcription of mRNA to protein synthesis.[49-52] Human CNOT4 regulates IFNγ production and IL4-dependent STAT signaling activation in human cervical cancer (HeLa cells), and these processes are involved in the immune/stress response, stem cell maintenance, and cancer progression.[53] Notably, Buschauer and colleagues[54] recently reported a physical link between the Ccr4-Not complex and the ribosome and provided mechanistic insight into the coupling of decoding efficiency with mRNA stability. Therefore, both EXOSC3 and CNOT4 are component molecules that control the lifespan of a wide variety of RNAs, leading to the onset of various diseases. We here revealed that EXOSC3 and CNOT4 were also expressed in precancerous lesion of SSL and that the expression of EXOSC3 and MYD88 is likely to correlate with each other (Figure S5B). Of note, the expression of CNOT4 clearly increased in the SSL compared with the paired adjacent normal mucosa (Figure S5B), suggesting that CNOT4 might be dominant in controlling RNAs in SSL. We would like to clarify the role of CNOT4 in SSL in more detail in the future. In this study, we discovered that the overexpression of EXOSC3 and CNOT4 promoted the gene expression of *IFNGR1*, *MYD88*, *NFKB1A*, and *STAT3* in human colonic epithelial cells, probably by upregulating the lifespan and metabolism of various mRNAs, and induced the activation of ERK1/2 and JNK in 293F cells (Figure 7), which was linked to the development of CRC. During the chronic inflammation caused by ulcerative colitis, the colonic epithelium is almost entirely remodeled by changes in the expression of numerous genes, which may lead to the onset of clones harboring driver mutations.[31] Colorectal cancer has been suspected to be promoted by commensal bacteria or dysbiotic microbiomes via

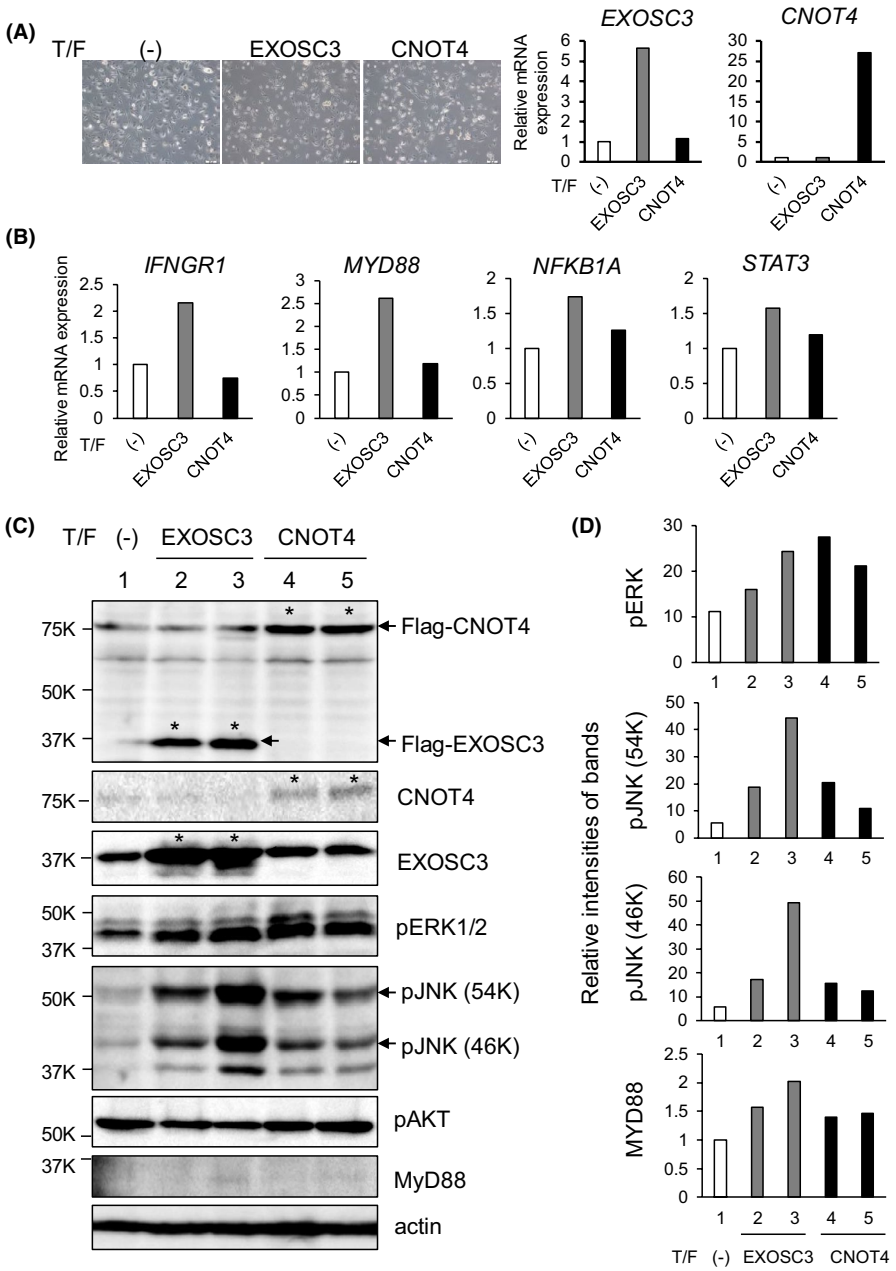


FIGURE 7 Overexpression of EXOSC3 and CNOT4 increased the expression of *IFNGR1*, *MYD88*, *NFKB1A*, and *STAT3* and led to the activation of JNK and ERK1/2. A and B, Human colonic epithelial cells (HCoEpiCs) were transiently transfected with expression plasmids coding human *EXOSC3* and *CNOT4*. Cell morphologies after 48 h of transfection are shown (left panels), and the gene expression of EXOSC3 and CNOT4 was examined by qRT-PCR (right graphs) (A). The expression levels of *IFNGR1*, *MYD88*, *NFKB1A*, and *STAT3* were examined by qRT-PCR (B). C and D, 293F cells were transiently transfected with expression plasmids producing human EXOSC3 and CNOT4. Cell lysates were subjected to immunoblotting with antibodies against the indicated proteins. Actin was used as a loading control. * Indicates the induced proteins (C). The band intensities of phospho-ERK, phospho-JNK (54 kDa band), phospho-JNK (46 kDa band), and MYD88 were measured by software and graphed (D)

dysbiosis, barrier failure, chronic inflammation, and bacterial genotoxicity.[55,56] The kinds of intestinal bacteria that enhance the expression levels of EXOS C3 and CNOT4 should be elucidated. Therefore, further investigation should be performed to verify the relationship between the promotion of RNA-controlling factors and the development of colon cancer.

In summary, we revealed that EXOSC3 and CNOT4 would be potential predictive markers of colon cancer and may contribute to the onset by stabilizing RNAs including *MYD88*. Individuals with high expression should be followed up to enable early detection of CRC. Furthermore, novel molecular therapeutics targeting these molecules would be a valuable strategy to prevent the onset of CRC, which may introduce a new era in preventive medicine for cancer.

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DISCLOSURE

The authors declare no competing financial interests.

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SUPPORTING INFORMATION

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