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ADAR1-high tumor-associated macrophages induce drug resistance and are therapeutic targets in colorectal cancer

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Abstract

Background Colorectal cancer (CRC) is considered the third most common type of cancer worldwide. Tumor-associated macrophages (TAMs) have been shown to promote drug resistance. Adenosine-to-inosine RNA-editing, as regulated by adenosine deaminase acting on RNA (ADAR), is a process that induces the posttranscriptional modification of critical oncogenes. The aim of this study is to determine whether the signals from cancer cells would induce RNA-editing in macrophages.

Methods The effects of RNA-editing on phenotypes in macrophages were analyzed using clinical samples and in vitro and in vivo models.

Results The intensity of the RNA-editing enzyme ADAR1 (Adenosine deaminase acting on RNA 1) in cancer and mononuclear cells indicated a strong positive correlation between the nucleus and cytoplasm. The ADAR1-positive mononuclear cells were positive for CD68 and CD163, a marker for M2 macrophages. Cancer cells transport pro-inflammatory cytokines or ADAR1 protein directly to macrophages via the exosomes, promoting RNA-editing in AZIN1 (Antizyme Inhibitor 1) and GLI1 (Glioma-Associated Oncogene Homolog 1) and resulting in M2 macrophage polarization. GLI1 RNA-editing in the macrophages induced by cancer cells promotes the secretion of SPP1, which is supplied to the cancer cells. This activates the NFκB pathway in cancer cells, promoting oxaliplatin resistance. When the JAK inhibitors were administered, oncogenic RNA-editing in the macrophages was suppressed. This altered the macrophage polarization from M2 to M1 and decreased oxaliplatin resistance in cancer cells.

Conclusions This study revealed that ADAR1-high TAMs are crucial in regulating drug resistance in CRC and that targeting ADAR1 in TAMs could be a promising treatment approach for overcoming drug resistance in CRC.

Keywords RNA-editing, Macrophage, Chemoresistance, Biomarker, Colorectal cancer

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Background

Colorectal cancer (CRC) is considered a global health concern, currently ranking as the third most common type of cancer worldwide. With an estimated 3.2 million new cases and 1.6 million deaths due to CRC by 2040, mainly in developed countries, further research is needed to gain a deeper understanding of the disease and develop effective treatment options [1]. In the treatment of CRC with distant metastases, implementing an effective drug therapy is the first step of treatment. In particular, oxaliplatin has been widely used as a critical drug in treating CRC following the MOSAIC trial. In metastatic CRC, oxaliplatin has become an essential part of the standard of care as a member of FOLFOX therapy [2–4]. However, the average duration of response to oxaliplatin is approximately 6–8 months, which is not always sufficient. Various efforts are being made to extend the duration of response, one of which being the use of molecular-targeted agents. Recently, the results of the PARADIGM study revealed that the use of anti-EGFR antibody drugs in combination with oxaliplatin prolongs the overall survival in KRAS wild-type CRC [5]. The aim of this study is to contribute further findings and potentially revolutionize the treatment of CRC by addressing the problem of oxaliplatin resistance.

The role of macrophages is essential when considering the resistance to anticancer drugs. TAMs have been shown to promote drug resistance [6–8] and have an M2-type polarity, which leads to cancer cell proliferation, angiogenesis, immunosuppression, and drug resistance. Certain strategies are being assessed to take advantage of this and enhance antitumor immune responses by reprogramming the M2 macrophages to the M1 type [9]. However, it is too complex to be explained by simple dualism since some macrophages have intermediate characteristics between M1 and M2 [10, 11]. Macrophages tend to flexibly change their functions in response to various signals in the cancer microenvironment, leading to several challenges in research.

However, we have investigated the significance of RNA-editing in CRC, which is a mechanism that alters the protein structure by changing only the RNA sequence without changing the DNA sequence, resulting in changes in the cell phenotype [12, 13]. The most famous RNA-editing enzyme is ADAR1, which is responsible for A to I editing that converts adenosine-to-inosine. We have also previously investigated the pro-tumorigenic role of RNA-editing in cancer cells and cancer-associated fibroblasts. The RNA-editing enzyme ADAR1 is highly expressed in CRC; the higher the RNA-editing, the poorer the prognosis [13]. Moreover, cancer cells induce RNA-editing in fibroblasts via humoral factors, enhancing the invasive potential of cancer-associated

fibroblasts [14]. Thus, it is clear that RNA-editing modifies the cell properties to a higher degree of malignancy in the cancer cells and fibroblasts that comprise the CRC microenvironment.

The present study hypothesized that a similar event may occur in TAMs and that signals from cancer cells would induce RNA-editing in TAMs, altering their properties and phenotypes in favor of the cancer cells. We focused on AZIN1 (Antizyme Inhibitor 1) and GLI1 (Glioma-Associated Oncogene Homolog 1) in particular as targets of the RNA editing enzyme ADAR1. We would like to show that the upregulation of ADAR1 in macrophages modifies their function via RNA editing of AZIN1 and GLI1, followed by oxaliplatin resistance in CRC. Blocking the M2 shift of macrophages via RNA editing may be able to reverse resistance to oxaliplatin and revolutionize drug therapy for CRC.

Methods

Patients and sample collection

Among the CRC patients who had their primary tumors resected at Okayama University Hospital between January 2011 and December 2023, a total of 151 CRC patients with distant metastasis were included in the analysis. Moreover, the Institutional Review Board of Okayama University Hospital approved the specimen collection and studies (1903–037). Overall survival was defined as the number of days from the time of confirmation of distant metastasis. Censoring was defined as the last day of confirmed survival by direct or telephone consultation. The efficacy of chemotherapy was evaluated according to RECIST v1.1 (Response Evaluation Criteria in Solid Tumors). Clinical samples were collected at the time of resection of the primary tumor. The clinicopathological characteristics are shown in Table 1.

Immunohistochemistry (IHC) analysis

The paraffin-embedded sections were deparaffinized using xylene and ethanol. Endogenous peroxidase activity was eliminated with H₂O₂. Anti-ADAR1 (1:500, ab168809, Abcam, Cambridge, UK) was the primary antibody.

IHC staining interpretation

The immunoreactive score (IRS) was used to assess the staining intensity in immunostaining. The IRS is a composite score that is commonly used in both clinical practice and translational research. The IRS is a total score for the distribution and intensity of immunostaining [15]. Specifically, the PP (proportion of positive cells) was assessed on a scale of 0–4 (0, 0%; 1, 1%–25%; 2, 26%–50%; 3, 51%–75%; 4, 76%–100%). The SI (staining intensity) was evaluated on a scale of 0–3 (0, negative; 1, weak; 2,

Table 1 Characteristics of the patients and ADAR1 expression in cancer cells

		ADAR1 (nucleus)			ADAR1 (cytoplasm)		
		Low	High	P value	Low	High	P value
Age	≤68 (Median)	43	38	0.580	42	39	0.688
	> 68 (Median)	34	36		34	36	
Gender	M	45	42	0.834	48	39	0.165
	F	32	32		28	36	
Stage IV or recurrent	Stage IV	49	53	0.295	48	54	0.246
	Recurrent	28	21		28	21	
Location	Right	17	24	0.153	16	25	0.090
	Left	60	50		60	50	
Colon or rectum	Colon	42	42	0.785	37	47	0.084
	Rectum	35	32		39	28	
CEA (ng/ml)	≤5.0	22	20	0.832	20	22	0.679
	> 5.0	55	54		56	53	
CA19-9 (U/ml)	≤37	51	42	0.231	48	45	0.690
	> 37	26	32		28	30	
Neoadjuvant chemotherapy	YES	49	43	0.486	52	40	0.057
	NO	28	31		24	35	
Pathology	Differentiated	65	51	0.024	63	53	0.075
	Undifferentiated	12	23		13	22	
Lymph node metastasis	Absent	30	8	0.000	28	10	0.001
	Present	47	66		48	65	
Number of lymph node metastases	≤4	58	38	0.002	55	41	0.024
	> 4	19	36		21	34	
Liver metastasis	Absent	15	13	0.762	15	13	0.704
	Present	62	61		61	62	
Number of liver metastasis	≤3	57	42	0.026	54	45	0.153
	> 3	20	32		22	30	
Type of liver metastasis	Synchronous	38	46	0.232	39	45	0.547
	Metachronous	24	15		22	17	
RAS mutation	Mutant	26	27	0.726	25	28	0.568
	Wild	51	47		51	47	

moderate; 3, strong). The IRS score is calculated by multiplying the PP and SI. Three blinded investigators visually evaluated this staining level, while the mean value was used as the evaluation value.

Immunofluorescence (IF)

Paraffin sections fixed with paraformaldehyde were deparaffinized with xylene and ethanol. Peroxidase blocking was performed with hydrogen peroxide, and antigen activation was performed with the manufacturer's recommended activation solution. The following antibodies were used: anti-CD68 (1:100, ab277276, Abcam, Cambridge, UK), anti-CD163 (1:100, ab281746, Abcam), and anti-ADAR1 (1:100, ab269823, Abcam). Observations were carried out using a confocal laser scanning microscope (LSM780, Carl ZEISS, Oberkochen, Germany) at

200× magnification, in at least three independent fields of view.

Cell culture

Of CRCs, 85% are microsatellite stable, and 15% are microsatellite unstable. Therefore, we decided to use HT29 as a representative of the microsatellite stable group and HCT116 as a representative of the microsatellite unstable group. These cells have also been used in our previous papers [13]. HT29 and HCT116 were obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB Cell Bank, Tokyo, Japan), while THP-1 was obtained from the American Type Culture Collection (ATCC, Manassas, USA). The mechanism by which THP-1 cells are treated with PMA (Phorbol 12-myristate 13-acetate) to differentiate into macrophages has been

reported in various papers [16]. This mechanism relies on cell differentiation, mainly mediated by the protein kinase C (PKC) signaling pathway. This process proceeds as follows. PMA is a chemical that binds to the C1 domain of PKC and activates PKC. This activation activates transcription factors (e.g., AP-1 and NF- κ B) that promote differentiation, inducing macrophage-related genes. Based on previous studies, THP-1 was treated with 200 nM PMA (P8139, Sigma-Aldrich, St. Louis, USA) and differentiated into macrophages (dTHP-1) after 48 h of stimulation.

Small interfering RNA (siRNA) transfection

The siRNA oligonucleotides against ADAR1 (Silencer Select s1008 & s1009, Thermo Fisher Scientific, Waltham, USA) and negative controls (Silencer Select Negative Control #1, Thermo Fisher Scientific) were used. The oligonucleotides were transfected with Lipofectamine RNAiMAX Transfection Reagent (#13,778,030, Thermo Fisher Scientific) in accordance with the manufacturer's protocol. siRNA transfection was carried out as follows. We stimulated THP-1 cells with 200 nM PMA, allowing them to adhere to the bottom of the dish, and then cultured them for 2 days to stabilize them. We adjusted the concentration of siRNA targeting ADAR1 to 5 nM per well and then introduced it into THP-1 cells using Lipofectamine RNAiMAX.

Real-time q-PCR

Real-time q-PCR was used for gene expression analysis with the StepOne Real-Time PCR System (Applied Biosystems, Waltham, USA) and FAST SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, USA) as previously described [17, 18]. GAPDH was used as a normalization control. The relative expression of each mRNA was determined using the $\Delta\Delta C_t$ method. Supplementary Table 1 shows the primer sequences (i.e., ADAR, GAPDH, SPP1, BIRC3, NOS2, and ARG-1).

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

The degree of editing AZIN1 RNA was assessed using the RNA-editing site-specific quantitative polymerase chain reaction (RESS q-PCR) method as published previously [13, 19]. The key is the primer design. This is not our original technology but a technology designed by Dr. Crews [19]. They deliberately introduce a mismatch at the third base from the end of the wild type and edited type primers to make it easier to recognize the editing site at the end. In brief, specific primers for wild-type and edited AZIN1 sequences were designed. Based on the C_t values, the ratios between the edited and wild-type AZIN1 were measured using formula $2^{-(C_t \text{ Edited} - C_t \text{ Wild-type})}$. This

value is defined as the RNA editing ratio. Supplementary Table 1 shows the primer sequences for the PCRs.

Western immunoblotting

The proteins extracted from cells and extracellular vesicles (EVs; 50 μ g and 10 μ g, respectively) were electrophoresed on SDS–polyacrylamide gels and transferred to Hybond-polyvinylidene fluoride membranes (GE Healthcare UK, Amersham, UK). The membranes were reacted at a temperature of 4 °C overnight with the following primary antibodies: anti-ADAR1 (1:1000, ab168809, Abcam, Cambridge, UK), anti- β -actin (1:1000, #A5441, Sigma-Aldrich, St. Louis, USA), and anti-CD63 (1:1000, 10628D, Invitrogen, Carlsbad, USA).

Collection and tracking of EV

EVs were isolated by collecting the supernatant after culturing for 48 h in a medium that did not contain FBS and then performing ultracentrifugation [20]. After spinning down the cellular components, the supernatant was filtered through a 0.22 μ m filter and centrifuged at 100,000 g for 70 min at 4 °C. The pellet was washed with PBS, ultracentrifuged under the same conditions, and dissolved in PBS. Protein concentration was measured using the BCA assay according to the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, USA). EV size was measured using the DLS method with a Zetasizer Nano ZSP (Malvern Instruments, Malvern, UK). EV morphology and structure were visualized using a Transmission Electron Microscope (H-7560, Hitachi, Tokyo, Japan). For the experiment on uptake into cells, EVs were stained with PKH26 red fluorescent dye (MedChemExpress, Monmouth Junction, NJ, USA) and observed. EVs stained with PKH26 red fluorescent dye were added to dTHP-1 and observed using an inverted research microscope (IX83, Olympus Life Science, Tokyo, Japan) 24 h later.

XTT assays

The cells were seeded in 96-well plates at a density of 1.0×10^4 /well. The cell survival was measured using the Cell Proliferation Kit II (XTT, 11,465,015,001, ROCHE, Basel, Switzerland) in accordance with the manufacturer's protocol.

mRNA library construction and sequencing

1 μ g total RNA was used for the following library preparation. The poly(A) mRNA isolation was performed using Oligo(dT) beads. The mRNA fragmentation was performed using divalent cations and high temperatures. Priming was performed using Random Primers. First-strand cDNA and the second-strand cDNA were synthesized. The purified double-stranded cDNA was

then treated to repair both ends and add a dA-tailing in one reaction, followed by a T-A ligation to add adaptors to both ends. Size selection of Adaptor-ligated DNA was then performed using DNA Clean Beads. Each sample was then amplified by PCR using P5 and P7 primers, and the PCR products were validated. Then, libraries with different indexes were multiplexed and loaded on an Illumina HiSeq/ Illumina Novaseq/ MGI2000 instrument for sequencing using a 2×150 paired-end (PE) configuration according to the manufacturer's instructions.

RNA sequence data analysis

Quality control

In order to remove technical sequences, including adaptors, polymerase chain reaction (PCR) primers, or fragments thereof, and bases of lower quality than 20, pass filter data of fastq format were processed by Cutadapt (V1.9.1, phred cutoff: 20, error rate: 0.1, adapter overlap: 1 bp, min. length: 75, proportion of N: 0.1) to be high-quality clean data.

Alignment

Firstly, reference genome sequences and gene model annotation files of relative species were downloaded from genome websites such as UCSC, NCBI, and ENSEMBL. Secondly, Hisat2 (v2.0.1) was used to index the reference genome sequence. Finally, clean data were aligned to the reference genome via the software Hisat2 (v2.0.1).

Expression analysis

In the beginning, transcripts in fasta format are converted from a known gff annotation file and indexed properly. Then, with the file as a reference gene file, HTSeq (v0.6.1) estimates gene and isoform expression levels from the pair-end clean data.

Gene set enrichment analysis (GSEA)

The normalized transcript reads (TPM) were used for downstream analysis. Gene set enrichment analysis was performed using GSEA software [21].

Intracellular polyamine detection

For the polyamine experiment, we followed the manufacturer's protocol. PolyamineRED (Funakoshi, Tokyo, Japan) was added to the newly prepared culture medium at a final concentration of 10–30 µM. The culture medium of the cultured cells was removed, and the cells were washed twice with PBS. The culture medium containing PolyamineRED was added. The cells were cultured for 10 min and washed three times with PBS. After paraformaldehyde fixation, the cells were stained with a nuclear stain and observed under a fluorescence microscope.

Flow cytometry analysis

We prepared dTHP-1 with and without adding a cancer cell culture medium. After the total cell count was determined and the cell viability was confirmed, the cells were washed, fixed with 4% paraformaldehyde, and allowed to react with the antibody for 30 min in the dark at room temperature. The antibody was used at the concentration recommended by the manufacturer. The dTHP-1 cells were stained with the following antibodies and analyzed via flow cytometry (BD FACSLyric, BD Biosciences, San Jose, USA). The following antibodies were used: anti-CD163 (326,506, BioLegend, San Diego, USA), anti-CD80 (FITC, 305,206, BioLegend, San Diego, USA), and isotype-matched control antibodies.

In vivo experiment

The number of cells used in the animal experiments was determined by referring to previous reports [22]. During xenograft formation, the 5.0×10^6 HCT116 cells and 1.0×10^6 PMA-treated THP-1 (dTHP-1) were mixed and injected subcutaneously and dorsally into the BALB/c nude mice. The frequency of oxaliplatin administration was based on previous reports [23]. Oxaliplatin (200 µg/body) was administered intraperitoneally every four days, while a JAK inhibitor (filgotinib, 200 µg/body) was administered intraperitoneally every two days. In the oxaliplatin administration experiment in Fig. 4, 7 mice were used in each group. In the JAK inhibitor administration experiment in Fig. 5, 5 mice were used in each group.

Public database analysis

Data on the reported cytokines in the colon cancer cell lines (CACO2, RKO, HT29, and HCT116) were extracted from the Cancer Cell Line Encyclopedia (CCLE). The ADAR expression and genes involved in the JAK/STAT pathway were also compared on cBioPortal using a CRC microarray dataset (TCGA, Firehose Legacy). The data analysis of the macrophage polarity changes due to ADAR1 deficiency was performed by extracting the data from GSE184323.

Cytokine array

HT29 and HCT116 cells were incubated with FBS-free medium for 48 h. Culture medium was collected and subjected to human cytokine antibody array (ab133997, Abcam) according to the manufacturer's instructions. Briefly, the arrays were first blocked with blocking buffer for 30 min, then incubated with 1 ml culture medium at 4 °C overnight, followed by incubation with Biotin-conjugated anticytokines cocktail at 4 °C overnight. The membranes were washed and then incubated with 2 ml

HRP-conjugated streptavidin at 4 °C overnight. Chemiluminescence detections were performed using a CCD camera with the imaging system.

Statistical analysis

All statistical analyses were performed using STATA (version 17.0 SE-Standard Edition). The survival analysis was conducted using the log-rank test. The correlation of the immunostaining data was analyzed using Spearman's rank correlation coefficient. The two groups were compared using the Mann–Whitney U test, and statistical significance was set at a p value of less than 0.05.

Results

ADAR1-high macrophages have been discovered in colorectal tissue

In our previous studies, immunostaining for the RNA-editing enzyme ADAR1 in CRC was performed. Consistent with our previous findings, the ADAR1 expression levels in the present study were assessed using a 4-point scale (from 0 to 3) and scored using the IRS (Fig. 1A). Table 1 shows the relationship between the ADAR1 expression and clinicopathological features in the clinical cohort. Among the CRC patients who had their primary tumors removed at Okayama University Hospital between January 2011 and December 2023, 151 patients with distant metastases were included in the analysis. In these CRC patients, two groups were established: the ADAR1 high group and the ADAR1 low group, with the median IRS of ADAR1 in the primary tumor as the cutoff. The high ADAR1 expression in the cancer cells was associated with undifferentiated type (nucleus ($p=0.024$)), positive lymph node metastasis (nucleus ($p=0.000$) and cytoplasm ($p=0.001$)), high number of lymph node metastases (nucleus ($p=0.002$) and cytoplasm ($p=0.024$)), and high number of liver metastases (nucleus ($p=0.026$)). Next, we analyzed which cells express ADAR1. Immunostaining for ADAR1 in the primary CRC tissue is positive for ADAR1 in the epithelial cancer cells and mononuclear cells (Fig. 1B). Incidentally, mononuclear cells and other cells were confirmed by visual inspection. In our previous reports, we have

stained ADAR1 in the cancer cells and cancer-associated fibroblasts. However, the ADAR1 expression in the mononuclear cells was not analyzed. Therefore, in the present study, the ADAR1 expression in the mononuclear cells was analyzed to clarify their clinicopathological significance. The two isoforms of ADAR1 are as follows: p110, which is strongly expressed in the nucleus, and p150, which is strongly expressed in the cytoplasm. For this reason, the expression intensity of ADAR1 in the nucleus and cytoplasm were measured separately. Both patients with high nuclear and cytoplasmic ADAR1 expression showed a trend toward shorter survival after distant metastasis (nucleus ($p=0.0026$) and cytoplasm ($p<0.001$); Fig. 1C). This finding is consistent with our previous report indicating that CRCs with high ADAR1 expression are highly malignant [12, 13]. Conversely, the mononuclear cells in cancer tissues were also assessed. We created a graph with ADAR1 IRS in macrophages on the Y-axis and ADAR1 IRS in the nucleus and cytoplasm of cancer cells on the X-axis. Each dot represents an individual patient. The intensity of ADAR1 staining in cancer cells and ADAR1 immunostaining in the mononuclear cells also revealed a strong positive correlation (between the nucleus of cancer cells and mononuclear cells: $\rho=0.74$, $p<0.001$; between the cytoplasm of cancer cells and mononuclear cells: $\rho=0.65$, $p<0.001$; Fig. 1D). In particular, the CRC patients with mononuclear cells that have a higher expression of ADAR1 in the cancer tissue than in the surrounding cancer area revealed a trend toward shorter survival after distant metastasis ($p=0.032$; Fig. 1E). We aimed to determine the type of cell population these ADAR1-expressing mononuclear cells represent. Thus, we performed fluorescence multiplex immunostaining on the CRC tissue. We observed that the ADAR1-positive mononuclear cells were positive for CD68, which is considered a marker for macrophages (Fig. 1F, G). Incidentally, the analysis of the clinical data indicated that high ADAR1 expression in the macrophages was associated with undifferentiated type (tumor center ($p=0.024$)), lymph node metastasis (tumor edge ($p=0.004$)), high number of lymph node metastases (tumor center ($p=0.041$) and tumor edge ($p=0.002$)),

(See figure on next page.)

Fig. 1 ADAR1-high macrophages are present in colon cancer tissue. **A** The ADAR1 expression levels were assessed using a 4-point scale (from 0 to 3) and scored using the IRS. ADAR1 expression is visualized as brown staining, resulting from DAB (3,3'-diaminobenzidine) precipitation following HRP-conjugated secondary antibody reaction. Hematoxylin counterstaining was performed to visualize nuclei. **B** Immunostaining for ADAR1 in the primary CRC tissue indicated a positivity for ADAR1 in the epithelial cancer cells and mononuclear cells. Incidentally, mononuclear cells and other cells were confirmed by visual inspection. **C** Both the patients with high nuclear and cytoplasmic ADAR1 expression showed a trend toward shorter survival after distant metastasis. **D** The ADAR1 IRS in the cancer and mononuclear cells revealed a strong positive correlation between the nucleus and the cytoplasm. **E** The CRC patients with ADAR1-high mononuclear cells revealed a trend toward shorter survival after distant metastasis. **F, G** The ADAR1-high mononuclear cells were positive for CD68, which is considered a marker for macrophages. IHC, immunohistochemistry; IRS, immunoreactive score; CRC, colorectal cancer

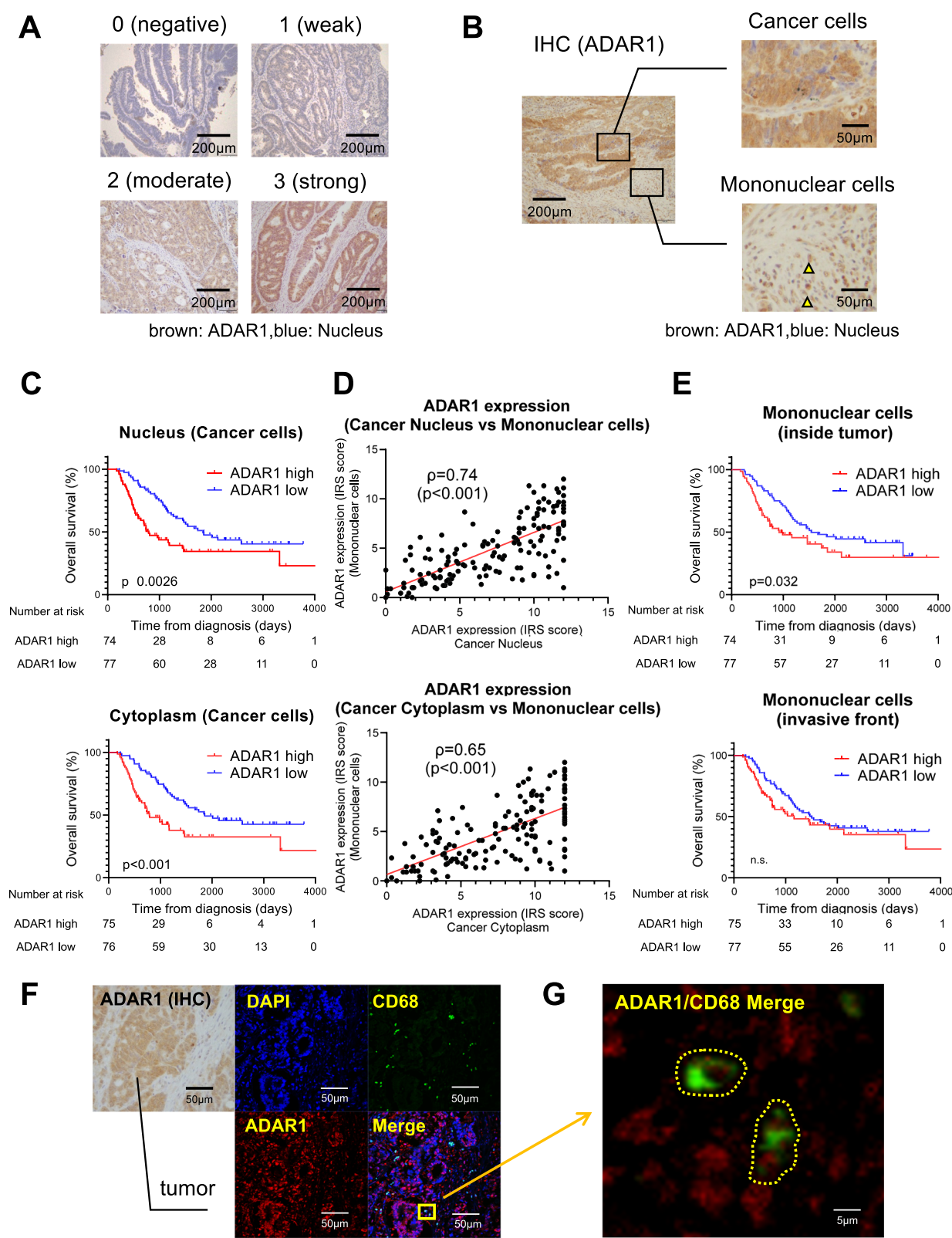


Fig. 1 (See legend on previous page.)

liver metastasis (tumor center ($p=0.017$)), high number of liver metastases (tumor edge ($p=0.026$)), and synchronous liver metastasis (tumor center ($p=0.030$); Table 2). CRCs with ADAR1-high macrophages are more likely to form liver metastases (multivariate ($p=0.025$); Table 3). Based on these solid clinical facts, we hypothesized that “ADAR1-high TAMs” may contribute to the progression of CRC and can be a therapeutic target in CRC.

CRC cells promote ADAR1 expression and RNA-editing in macrophages

First, we analyzed how the expression of the RNA-editing enzyme ADAR1 is induced in macrophages in an in vitro model. The culture medium (CM) of the colon cancer cell lines (HCT116 and HT29) was added to PMA-treated THP-1, a macrophage model (Fig. 2A). Moreover, the RNA and protein expression levels of ADAR1 were

increased in dTHP-1 (HCT116 CM ($p=0.009$) and HT29 CM ($p=0.002$); Fig. 2B). Since cancer cells tend to secrete pro-inflammatory cytokines, including interleukin family cytokines that induce ADAR1 expression, cytokine stimulation from cancer cells may cause ADAR1 transcriptional activity in dTHP-1. To investigate this, we analyzed cytokines secreted from cancer cells. We extracted data on cytokines reported for colon cancer cell lines (CACO2, RKO, HT29, HCT116) from the Cancer Cell Line Encyclopedia (CCLE, <https://sites.broadinstitute.org/ccle>). As a result, we found that the expression of pro-inflammatory cytokines such as IL-1 was indeed increased. We also performed a cytokine array using the culture supernatants of HCT116 and HT29 cells. We were able to confirm that the culture supernatants of these cancer cell lines contained pro-inflammatory cytokines such as IL-1, IL-2, IL-5, IL-8, IFN- γ , TNF- α ,

Table 2 Characteristics of the patients and ADAR1 expression in macrophages

		ADAR1 (tumor center)			ADAR1 (tumor edge)		
		Low	High	P value	Low	High	P value
Age	≤68 (Median)	44	37	0.379	42	39	0.820
	> 68 (Median)	33	37		35	35	
Gender	M	41	46	0.268	50	37	0.063
	F	36	28		27	37	
Stage IV or recurrent	Stage IV	47	55	0.081	48	54	0.163
	Recurrent	30	19		29	20	
Location	Right	17	24	0.153	16	25	0.072
	Left	60	50		61	49	
Colon or rectum	Colon	40	44	0.353	38	46	0.113
	Rectum	37	30		39	28	
CEA (ng/ml)	≤5.0	23	19	0.565	20	22	0.607
	> 5.0	54	55		57	52	
CA19-9 (U/ml)	≤37	49	44	0.598	51	42	0.231
	> 37	28	30		26	32	
Neoadjuvant chemotherapy	YES	51	41	0.173	49	43	0.486
	NO	26	33		28	31	
Pathology	Differentiated	65	51	0.024	63	53	0.138
	Undifferentiated	12	23		14	21	
Lymph node metastasis	Absent	24	14	0.083	27	11	0.004
	Present	53	60		50	63	
Number of lymph node metastases	≤4	55	41	0.041	58	38	0.002
	> 4	22	33		19	36	
Liver metastasis	Absent	20	8	0.017	17	11	0.254
	Present	57	66		60	63	
Number of liver metastasis	≤3	56	43	0.059	57	42	0.026
	> 3	21	31		20	32	
Type of liver metastasis	Synchronous	36	48	0.030	38	46	0.268
	Metachronous	21	18		22	17	
RAS mutation	Mutant	26	27	0.726	27	26	0.993
	Wild	51	47		50	48	

Table 3 Multivariate analysis for the prediction of liver metastasis

	Univariate			Multivariate		
	OR	95% CI	P value	OR	95% CI	P value
Age (> 68: median)	1.190	0.520–2.723	0.681			
Gender (female)	0.685	0.301–1.561	0.368			
Side (left)	1.347	0.553–3.279	0.512			
Location (rectum)	1.078	0.471–2.470	0.858			
CEA (> 5.0 ng/ml)	4.974	2.095–11.810	0.000	4.664	1.856–11.720	0.001
CA19-9 (> 37U/ml)	2.125	0.840–5.372	0.111	1.376	0.502–3.775	0.535
Histological type (poorly)	1.131	0.419–3.057	0.808			
Neoadjuvant chemotherapy	1.771	0.724–4.334	0.211			
ADAR1 expression (cancer, nucleus)	1.135	0.499–2.584	0.762			
ADAR1 expression (cancer, cytoplasm)	1.172	0.515–2.669	0.704			
ADAR1 expression (macrophages, tumor center)	2.894	1.185–7.071	0.020	2.926	1.144–7.480	0.025
ADAR1 expression (macrophages, tumor edge)	1.622	0.703–3.747	0.257			
RAS mutation	1.792	0.707–4.543	0.219			

and TNF- β (Fig. 2C). These pro-inflammatory cytokines activate the JAK/STAT pathway leading to ADAR1 [24]. In addition, IFN- γ and TNF- α can directly induce ADAR1 [25]. It seems that direct and indirect pro-inflammatory cytokine stimulation from cancer cells promotes ADAR1 expression in macrophages.

Conversely, recent studies have reported that the protein ADAR1, an RNA-editing enzyme, is secreted directly from cancer cells and taken up by surrounding cells, thereby altering their phenotype [26]. We hypothesized that this mechanism might be valid in the crosstalk between cancer cells and macrophages. We aimed to analyze what contains the proteins that move between cells. When the exosomes in the HCT116 culture supernatant were labeled with red fluorescent EV tracking and added to dTHP-1, EV tracking was transferred to the dTHP-1 cytoplasm (Fig. 2D). The exosome-mediated intercellular communication pathway from the cancer cells to the macrophages is present. Furthermore, we aimed to determine if this included ADAR1.

First, exosomes were recovered from the culture supernatants of the colon cancer cell lines HCT116 and HT29 using ultracentrifugation. The electron microscopy

and size measurements confirmed that the exosomes were recovered (Fig. 2E). The western blotting analysis revealed that these exosomes contain ADAR1 (Fig. 2F). When these exosomes were added to dTHP-1, the content of the ADAR1 protein increased in dTHP-1 (Fig. 2G). The ADAR1 in the cancer cells was proven to be transmitted to dTHP-1 via the exosomes.

ADAR1 reaching dTHP-1 increased the RNA-editing in dTHP-1, including AZIN1 (HCT116 EV ($p < 0.001$) and HT29 EV ($p < 0.001$)) and GLI1 (HCT116 EV ($p = 0.018$) and HT29 EV ($p = 0.055$); Fig. 2H). These results revealed that the cancer cells approach the macrophages at the RNA and protein levels and promote the expression of the RNA-editing enzyme ADAR1 in the macrophages, thereby promoting RNA-editing in AZIN1 and GLI1. Thus, we decided to analyze the phenotypic effect of the RNA-editing of AZIN1 and GLI1 on macrophages in the subsequent analyses.

ADAR1 promotes macrophage M2 shift via RNA-editing

First, we analyzed the properties of ADAR1-high macrophages in the CRC tissue. Macrophages are roughly classified as M1 and M2, wherein the M2 type reduces

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Fig. 2 ADAR1-high macrophages are induced by cancer cells. **A** The culture supernatants of colon cancer cell lines were added to dTHP-1. **B** The RNA and protein expression levels of ADAR1 were increased in dTHP-1. **C** Cancer cells tend to secrete pro-inflammatory cytokines that induce ADAR1 expression. **D** When exosomes in the HCT116 culture supernatant were labeled with red fluorescent EV tracking and added to dTHP-1, EV tracking was transferred to the dTHP-1 cytoplasm. **E** The results of the electron microscopy and size measurements confirmed that the exosomes were recovered. **F** The western blotting analysis revealed that these exosomes contain ADAR1. **G** When these cancer exosomes were added to dTHP-1, the content of the ADAR1 protein increased in dTHP-1. **H** ADAR1 reaching dTHP-1 increased the RNA-editing in dTHP-1, including AZIN1 and GLI1. PMA, phorbol 12-myristate 13-acetate; CM, culture medium; EV, extracellular vesicle. * $p < 0.05$. ** $p < 0.05$. *** $p < 0.001$



antitumor immunity. It is essential to determine under which side of the continuous M spectrum the ADAR1-high macrophages are classified. Multiplex fluorescent immunostaining was performed on the CRC tissues. Most CD68-positive macrophages are positive for ADAR1 and CD163, which is considered a marker for M2 macrophages ($p=0.038$; Fig. 3A). Similarly, the fluorescence multiplex immunostaining in 12 cases revealed that most ADAR1-positive cells were found in the CD163-positive M2 macrophages ($p=0.038$; Fig. 3B). Subsequently, we determined whether this polarity shift of TAMs toward M2 was due to ADAR1.

We added HCT116 and HT29 CM to dTHP-1. We observed a decrease in the transcriptional activity of the M1 marker NOS2 (HCT116 CM ($p=0.0018$) and HT29 CM ($p<0.001$)) and an increase in the transcriptional activity of the M2 marker ARG1 (HT29 CM ($p=0.0058$); Fig. 3C). Following the RNA analysis, we attempted to visualize, at the protein level, how the culture supernatant of cancer cells changes the polarization balance of macrophages using flow cytometry. We added cancer cell culture supernatant to PMA-treated THP-1 (dTHP-1) cells, which mimic macrophages, and observed the protein expression levels of M1 markers (CD80) and M2 markers (CD163). By adding cancer cell culture supernatant to dTHP-1, the signal waveform of CD80, an M1 marker, the signal waveform for the M1 marker CD80 shifted significantly to the left (Fig. 3D). Incidentally, macrophages can take on an intermediate state that has characteristics of both M1 and M2 at the same time in response to various signals from the environment [11]. This intermediate state indicates that the phenotype and function of macrophages change continuously, and it is thought that the ratio of M1 and M2 characteristics determines the state. Flow cytometry results show that the culture supernatant of cancer cells suppresses the M1 characteristics of macrophages and shifts them more towards M2. Subsequently, we determined whether this shift to M2 could be lifted when ADAR1 was suppressed in dTHP-1. A comprehensive analysis of the M1 and M2 markers in the bone marrow-derived macrophages from ADAR1-deficient mice revealed decreased M2 markers

CD163 and MRC1 and increased M1 markers CD80, CD86, and IRF5 (CD163, CD80, IRF5 ($p<0.05$), MRC1, CD86 ($p<0.01$); Fig. 3E). These results suggest that the regulation of the M1/2 polarity in macrophages is mediated by the RNA-editing enzyme ADAR1. It was also suggested that cancer cells may promote a shift to M2 through ADAR1 in macrophages.

As a step in which ADAR1 controls macrophage polarization, we would like to propose that ADAR1-mediated AZIN1 RNA editing, accumulation of ODC (ornithine decarboxylase), and shift to M2 are connected. There is a hotspot for A-to-I editing in the region encoding codon 367 (Ser) in AZIN1 mRNA, and when ADAR1 edits it, I (inosine) is recognized as G (guanosine), resulting in a Ser (S) to Gly (G) substitution. In particular, the p150 isoform of ADAR1 (induced by interferon stimulation) is known to have high editing activity for AZIN1 [12]. Normally, AZIN1 is an inhibitor of ODC, suppressing polyamine synthesis. However, AZIN1-Ser367Gly editing makes it harder to suppress ODC activity, and polyamine synthesis is enhanced. The edited form of AZIN1 has a strong inhibitory effect on Antizyme. As a result, ODC, which Antizyme was supposed to inhibit, accumulates, and polyamines increase [27]. In addition, previous reports have shown that ODC and polyamines suppress M1 markers and increase M2 markers in macrophages [28, 29]. We thought that if all of this could be connected, it should be possible to explain the process from the increase in ADAR1 in macrophages to the change in polarization to M2. The knockdown of ADAR1 in dTHP-1 reduces the RNA-editing of AZIN1 (HCT116 CM ($p<0.001$) and HT29 CM ($p=0.0033$); Fig. 3F). AZIN1 interacts with antizyme, an inhibitor of polyamine biosynthesis, and alleviates its inhibitory effect. This activates ODC, the main enzyme in the polyamine pathway, and promotes the production of polyamines. It is said that RNA editing enhances this effect [12]. With this in mind, we knocked down ADAR1 in dTHP1 cells and used PolyamineRED to label polyamines to check for any increase or decrease fluorescently. As a result, the red fluorescent signal for polyamines in the cells decreased in dTHP-1 cells where ADAR1 had been knocked down (Fig. 3G).

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Fig. 3 ADAR1 promotes the polarization of macrophages to M2. **A** Most CD68-positive macrophages are positive for ADAR1 and CD163, which is considered a marker for M2 macrophages. **B** The fluorescence multiplex immunostaining in 12 cases have revealed that most ADAR1-positive cells were found in CD163-positive M2 macrophages. **C** The HCT116 and HT29 cultures were added to dTHP-1, and a decrease in the transcriptional activity of the M1 marker NOS2 and increase in the transcriptional activity of the M2 marker ARG1 were observed. **D** The expression of the M1 marker CD80 also decreased at the protein level. **E** The comprehensive analysis of the M1 and M2 markers in the bone marrow-derived macrophages from ADAR1-deficient mice revealed a decrease in the M2 markers (i.e., CD163 and MRC1) and an increase in the M1 markers (i.e., CD80, CD86, and IRF5). **F** The knockdown of ADAR1 in dTHP-1 reduces the RNA-editing of AZIN1. **G** The polyamine accumulation in dTHP1 was also reduced. **H** The increase in ADAR1 shifts the polarity toward the M2 in macrophages. CM, culture medium; BMDM, bone-marrow-derived macrophages. * $p<0.05$. ** $p<0.05$. *** $p<0.001$

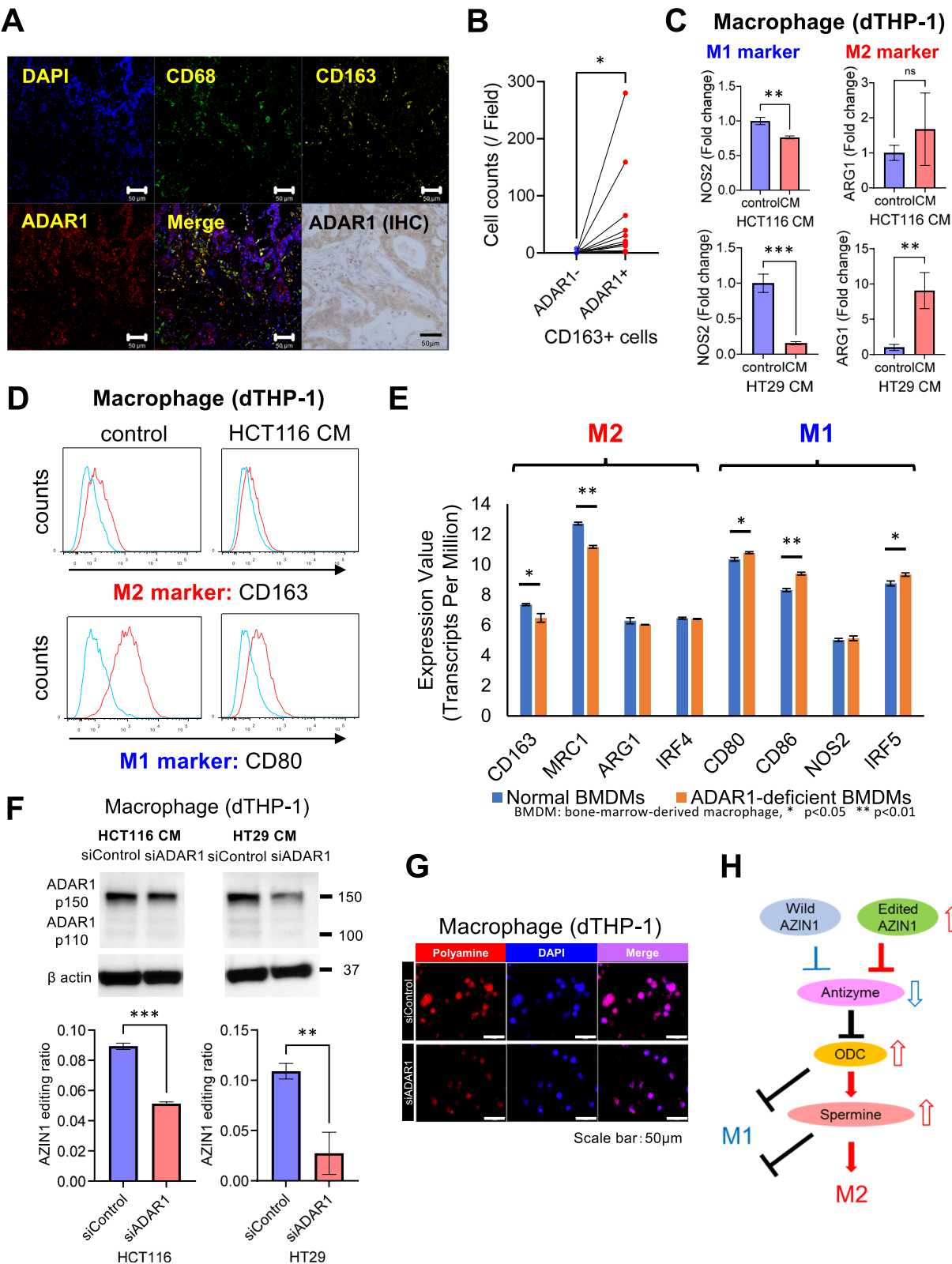


Fig. 3 (See legend on previous page.)

Based on these results, we aimed to explain the polarity change of macrophages to M2 in the CRC tissue. When ADAR1 is elevated in the macrophages upon stimulation from the cancer cells, the RNA-editing of AZIN1 is promoted. Edited AZIN1 encourages the accumulation of ODC and polyamines. Since previous reports have indicated that ODC and polyamines suppress the M1 markers and increase the M2 markers in the macrophages, we can explain the process from the increase in ADAR1 to the shift in polarity toward M2 [28] (Fig. 3H).

ADAR1-high macrophages induce oxaliplatin resistance in CRC

The findings of our study suggest that cancer cells may modify the macrophage phenotype via RNA-editing. Nevertheless, the classical classification cannot explain the macrophage function as M1 or M2. We propose a new concept of ADAR1-high TAMs as tumor-promoting macrophages. We also aimed to analyze the function of ADAR1-high macrophages and determine their significance.

Chemotherapy is essential in the treatment of CRC with distant metastases. In particular, first-line oxaliplatin regimens are often used, and the response to oxaliplatin defines the life expectancy. Patients who achieve a partial response to oxaliplatin-containing regimens are expected to survive longer than those who do not achieve a response ($p < 0.001$; Fig. 4A). We hypothesize that ADAR1-high TAMs in the CRC tissue may influence sensitivity to oxaliplatin. Interestingly, the ADAR1 expression in TAMs in the cancer tissue was higher in patients who did not respond to oxaliplatin ($p = 0.0046$; Fig. 4B). This led us to hypothesize that ADAR1-high TAMs may promote oxaliplatin resistance. HCT116 cells tend to induce oxaliplatin resistance when cocultured with dTHP-1 ($p = 0.0080$; Fig. 4C). However, when ADAR1 is knocked down first by siRNA in dTHP-1, the resistance of HCT116 to oxaliplatin is not induced ($p = 0.008$; Fig. 4D). This suggests that CRC cells need the help of ADAR1-high macrophages to become resistant to oxaliplatin. Similar results have been obtained in animal

models. Xenografts were made by mixing HCT116 and dTHP-1 in mice. The knockdown of ADAR1 in dTHP-1 enhanced the effect of oxaliplatin in inhibiting tumor growth ($p = 0.0040$; Fig. 4E). Thus, it is suggested that ADAR1-high TAMs induce oxaliplatin resistance in the CRC cells.

ADAR1-high macrophages promote SPP1 secretion via GLI1 RNA-editing

A comprehensive gene expression analysis was performed using a next-generation sequencer to characterize the ADAR1-high macrophages. siADAR1 was introduced into dTHP-1, and these are referred to as ADAR1-low macrophages. ADAR1-high macrophages are macrophages into which siControl was introduced. After thorough washing, co-culture was performed with cancer cells. ADAR1-high and ADAR1-low macrophages were compared using GSEA. In ADAR1-high macrophages, metastasis and chemotherapy resistance pathways were enriched (Fig. 4F). In particular, the expression of SPP1 (Secreted Phosphoprotein 1), which has recently attracted attention as a promoter of drug resistance, is upregulated. SPP1 is secreted by immune cells (e.g. macrophages and T cells) and is involved in the production of pro-inflammatory cytokines and the regulation of immune responses [30]. The immunostaining of the human clinical samples of CRC revealed a strong expression of SPP1 in ADAR1-high macrophages ($p < 0.001$; Fig. 4G). We confirmed this phenomenon in an in vitro model. Adding the HCT116 culture supernatant to dTHP1 increased the SPP1 expression ($p < 0.001$; Fig. 4H). The expression of SPP1 is regulated by GLI1 [31], which is known to be enhanced by RNA-editing [32]. GLI1 is a transcription factor in the Hedgehog signaling pathway, and it increases the transcriptional activity of SPP1 and other genes [33]. It has been reported that A-to-I editing in the coding region of GLI1 mRNA by ADAR1 results in an amino acid substitution of Arg701Gly (R701G) [32]. This edited form of GLI1 increases the Hedgehog signaling pathway and enhances the transcriptional

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Fig. 4 ADAR1-high macrophages cause oxaliplatin resistance. **A** Patients who achieve a partial response to oxaliplatin-containing regimens are expected to survive longer than those who do not achieve a response. **B** The ADAR1 expression in the macrophages of the cancer tissue was higher in patients who did not respond to oxaliplatin. **C** The HCT116 colon cancer cells also induce oxaliplatin resistance when co-cultured with the macrophage cell line dTHP-1. **D** When ADAR1 is knocked down first by siRNA in dTHP-1 cocultured with HCT116, oxaliplatin resistance is not induced. **E** The knockdown of ADAR1 in dTHP-1 enhanced the effect of oxaliplatin in inhibiting tumor growth. **F** In ADAR1-high macrophages, the pathways involved in metastasis and chemotherapy resistance were enriched. **G** The immunostaining of the human clinical samples of CRC revealed a strong expression of SPP1 in ADAR1-high macrophages. **H** The addition of the HCT116 culture supernatant to dTHP1 increased the SPP1 expression. **I** The addition of cancer cell culture supernatants to macrophages tends to increase the RNA-editing of GLI1 via elevated ADAR1 expression, producing SPP1. CM, culture medium; CRC, Colorectal cancer. * $p < 0.05$. ** $p < 0.05$. *** $p < 0.001$



activity of downstream genes such as SPP1 [34]. We also have already that adding cancer cell culture supernatants to macrophages increases the RNA-editing of GLI1 via elevated ADAR1 expression (Fig. 2H). Based on these findings, the following mechanisms can be deduced. First, the direct supply of cytokines or proteins from cancer cells increases the level of ADAR1 in macrophages. This promotes GLI1 editing and increases the efficiency of SPP1 production. SPP1 is a secreted protein, indicating that macrophages secrete SPP1. This acts on CRC cells to promote oxaliplatin resistance (Fig. 4I).

ADAR1-high macrophages induce oxaliplatin resistance via the NFκB pathway in colon cancer cells

We hypothesize that ADAR1-high macrophages induce oxaliplatin resistance in CRC cells through SPP1 secretion. To confirm this, we cocultured ADAR1-high and ADAR1-low macrophages with HCT116 and conducted a comprehensive genetic analysis of HCT116. The results revealed that the NFκB-related pathway was enriched in the HCT116 cocultured with ADAR1-high macrophages (Fig. 5A). Moreover, the direct addition of SPP1 to HCT116 also increased the level of BIRC3 in the NFκB pathway ($p=0.0012$; Fig. 5B). The NFκB pathway is an important pathway that promotes drug resistance [35]. In particular, BIRC3 is a gene that forms part of the NFκB pathway, and it has been reported that it causes resistance to oxaliplatin in CRC by inhibiting caspase 3 [36]. Since the expression of BIRC3 was increased in the CRC cell line HCT116 by the administration of SPP1, it was suggested that the mechanism by which ADAR1-high macrophages, which highly express SPP1, activate the NFκB pathway in CRC cells is due to the supply of SPP1 to CRC cells. This is shown in the schema as follows. First, the direct supply of cytokines or proteins from cancer cells increases ADAR1 in the macrophages. This promotes GLI1 RNA-editing and increases the efficiency of SPP1 production. Released SPP1 supports the activation of the NFκB pathway in cancer cells and induces drug resistance (Fig. 5C).

JAK inhibitor removes oxaliplatin resistance by suppressing ADAR1 expression and GLI1 RNA-editing in macrophages

Based on our previous findings, we aimed to construct a therapy targeting ADAR1-high macrophages to overcome oxaliplatin resistance in treating CRC. First, the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of high and low ADAR1-expressing CRCs revealed that the JAK/STAT pathway was activated in ADAR1-high CRCs (**Supplementary Fig. 1**). The gene expression analysis using the TCGA database also revealed a strong correlation between the ADAR1 and JAK family (JAK1, JAK2, JAK3) expressions (JAK1 ($\rho=0.34$, $p<0.001$), JAK2 ($\rho=0.49$, $p<0.001$), JAK3 ($\rho=0.44$, $p<0.001$); Fig. 5D). The JAK/STAT pathway is above ADAR1 and promotes ADAR1 expression [37]. Therefore, we investigated whether the JAK inhibitor could treat ADAR1-high CRC, which contains ADAR1-high TAMs.

First, we assessed the effect of the JAK inhibitor in an in vitro experimental system. An experimental system was set up in which a JAK inhibitor (filgotinib) was added to dTHP-1 when the cancer cell CM was added to dTHP-1 (Fig. 5E). The addition of HCT116 or HT29 CM with JAK inhibitors to dTHP1 decreased the ADAR1 expression (HCT116 CM ($p<0.001$), HT29 CM ($p=0.0039$); Fig. 5F) and GLI1 RNA-editing (HCT 116 CM ($p=0.0213$), HT29 CM ($p=0.0022$); Fig. 5G). Subsequently, in a mouse model, xenografts were created by mixing HCT116 and dTHP-1, and the differences in tumor growth in the oxaliplatin alone and oxaliplatin/JAK inhibitor combination groups were analyzed. The results revealed significant tumor growth inhibition in the oxaliplatin/JAK inhibitor group ($p=0.0197$; Fig. 5H). Since it was necessary to confirm whether the JAK inhibitor decreased ADAR1 in the tumor tissues, immunostaining for ADAR1 was performed, and the decreased expression of ADAR1 was observed in CRC cells or TAMs in the JAK inhibitor combination group (Fig. 5I). Thus, the combination of oxaliplatin and JAK inhibitors may be a promising treatment approach that can block ADAR1 and GLI1 editing in macrophages, which are responsible for oxaliplatin resistance.

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Fig. 5 ADAR1 target therapy using JAK inhibitors may overcome oxaliplatin resistance. **A** The NFκB-related pathway was enriched in the HCT116 cocultured with ADAR1-high macrophages. **B** The direct addition of SPP1 to HCT116 also increased the level of BIRC3 in the NF-κB pathway. **C** The released SPP1 from the macrophages promotes the activation of the NFκB pathway in cancer cells and induces drug resistance. **D** The gene expression analysis using the TCGA database also showed a strong correlation between ADAR1 expression and JAK family (JAK1, JAK2, JAK3) expression. **E** A JAK inhibitor (filgotinib) was added to dTHP-1 when the cancer cell CM was added to dTHP-1. **F** The addition of the HCT116 culture supernatant or HT29 culture supernatant with JAK inhibitor to dTHP1 decreased the ADAR1 expression. **G** The addition of the HCT116 or HT29 CM with JAK inhibitor to dTHP1 decreased the GLI1 RNA-editing. **H** Significant tumor growth inhibition was observed in the oxaliplatin/JAK inhibitor group. **I** The decreased expression of ADAR1 was observed in the tumor tissues in the JAK inhibitor combination group. CM, culture medium; JAKi, JAK inhibitor. * $p<0.05$. ** $p<0.05$. *** $p<0.001$

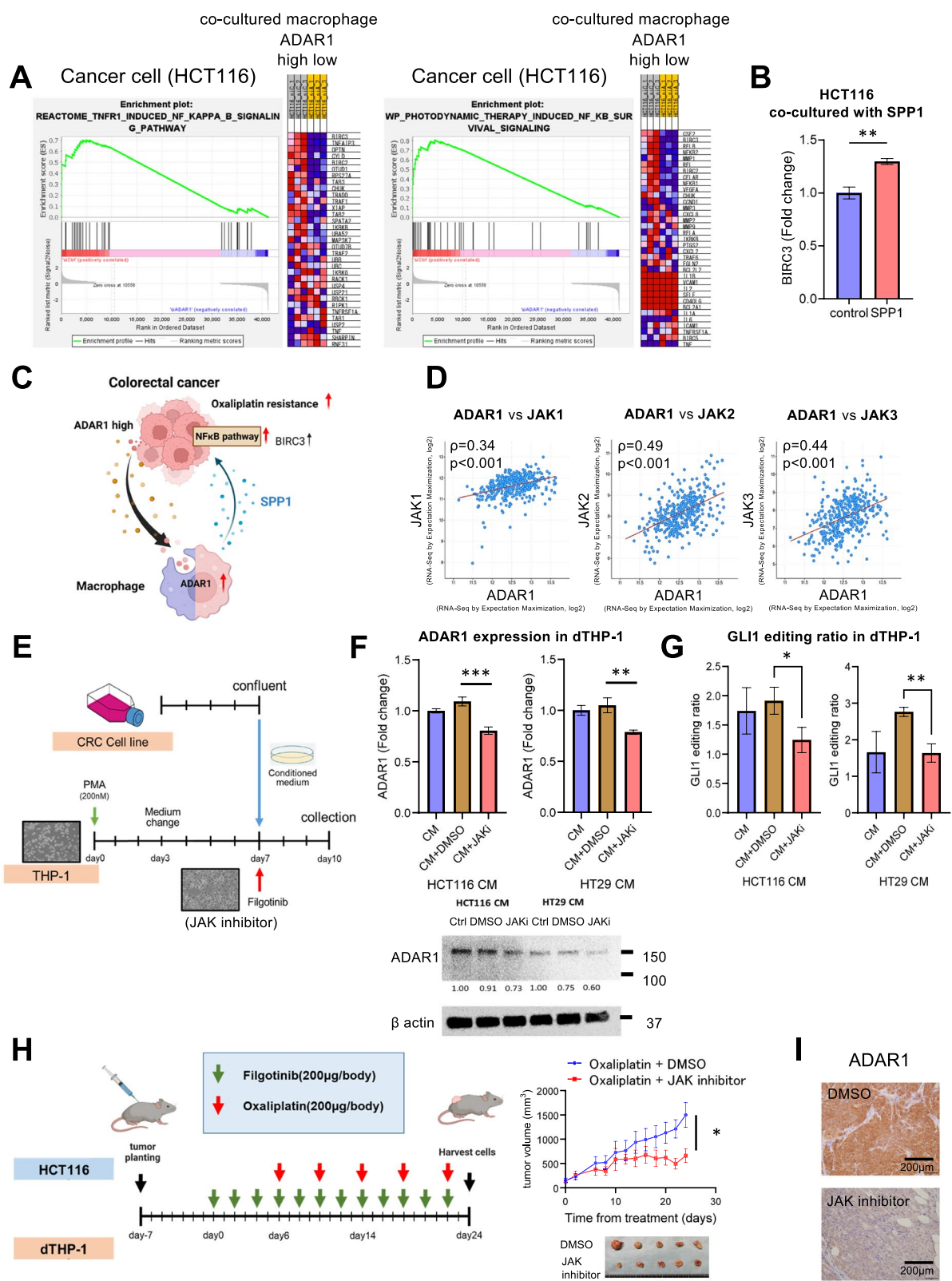


Fig. 5 (See legend on previous page.)

Discussion

The relationship between macrophages and chemotherapy resistance has been recognized in the mid to late 2000s [38]. The possible mechanisms include the secretion of cytokines and growth factors, immunosuppression, tumor microenvironment modification, and drug efflux acceleration. Some studies aimed to target macrophages, which promote drug resistance, for cancer treatment. The main one is inhibiting tumor growth by reducing macrophage density or altering macrophage polarity, but this has not yet led to actual therapy [6–8]. We suggested that it might be necessary to first understand the characteristics of TAMs from a different perspective. We have decided to apply the RNA-editing analysis as a tool for this purpose.

In this study, we have shown that RNA-editing plays an essential role in TAMs. First, cancer cells tend to promote the macrophage expression of ADAR1, which facilitates RNA-editing. Two patterns were considered: the cytokine supply from cancer cells and direct supply of ADAR1 protein. Second, ADAR1-high TAMs induced by cancer cells have an M2-like character and promote SPP1 secretion via the RNA-editing of GLI1. Third, SPP1 secreted from ADAR1-high macrophages activates the NF κ B pathway in cancer cells and induces oxaliplatin resistance. Fourth, the induction of ADAR1-high TAMs can be inhibited by the JAK inhibitors. These results revealed that the administration of the JAK inhibitors in CRC therapy can attenuate the tumor-promoting effects of ADAR1-high macrophages and oxaliplatin resistance. Since this study focuses on the role of ADAR1 in the macrophages in tumor tissues, we also considered a report of a mouse model in which the ADAR1 in the macrophages is knocked out and treated with interferon (IFN)- γ therapy [39]. The loss of ADAR1 in macrophages leads to the differential secretion of critical cytokines: it inhibits the translation of CCL20, GDF15, IL-18BP, and TIM-3 by inducing PKR/EIF2 α signaling but increases the secretion of IFN- γ . Finally, the knockout of ADAR1 induced antitumor immunity by enhancing the cytotoxicity of CD8 $^{+}$ T cells [39]. Thus, the fact that the ADAR1 in the macrophages suppresses antitumor immunity suggests that ADAR1 is essentially involved in cancer cells and other cells that comprise the cancer microenvironment. However, further analysis of RNA-editing, which is the original function of ADAR1, is needed to confirm our findings. Therefore, in this study, we aimed to investigate the role of ADAR1-high macrophages in CRC from a clinical perspective in relation to RNA-editing. Our study is the first to report that ADAR1-high TAMs are induced by tumor cells to cause drug resistance.

However, the fundamental question is how to suppress ADAR1. 8-Azaadenosine and 8-chloroadenosine are classically known ADAR1 inhibitors, but their ADAR1-independent cytotoxicity makes their clinical use difficult [40]. Recently, the use of rebecsinib, which suppresses splicing to the oncogenic ADAR1p150 isoform, was investigated. It inhibits the self-renewal of ADAR1p150-driven leukemia stem cells, while sparing the normal hematopoietic stem cells [41]. However, it will take some time to complete the clinical application. Thus, ADAR1-specific inhibitors have been considered for treatment, but cancer patients cannot immediately benefit from them. Considering this, we decided to assess the effectiveness of JAK inhibitors.

Preclinical studies in solid tumor cell line models have shown that JAK inhibitors decrease STAT activation, cell proliferation, and cell survival, leading to tumor growth inhibition [42]. However, the JAK inhibitors did not result in favorable results in the clinical trials for solid tumors. NCT02119676 was a study on ruxolitinib in CRC patients [43]. This study aimed to determine whether the combination treatment of ruxolitinib and regorafenib is safe in treating metastatic CRC. Although adding ruxolitinib to regorafenib did not raise increased safety concerns in patients with relapsed/refractory metastatic CRC, this combination treatment did not improve survival. One of the several possible reasons why the JAK inhibitor was deemed ineffective is that the patients had already been administered with multiple anticancer drugs. JAK inhibitors are compatible with oxaliplatin, as we have shown in the present study. However, better results might have been achieved if the combination of oxaliplatin and JAK inhibitors had been used in the first- or second-line therapy. We also plan to conduct a clinical trial of the combination treatment of oxaliplatin and JAK inhibitors in the future. In particular, filgotinib has already been used in treating rheumatoid arthritis, and its safety has already been proven. From the viewpoint of drug repositioning, filgotinib is considered an easy-to-use drug [44].

The limitations of this study include the fact that it was a single-center, backward-looking validation and that only one JAK inhibitor was used. Thus, we aim to conduct a prospective intervention study at other facilities. Additionally, the ADAR1 gene has two isoforms, p150 and p110. The p150 isoform is mainly found in the cytoplasm. It has been attracting attention as a target for cancer treatment because it creates an immunosuppressive environment in tumors through RNA editing [45]. On the other hand, p110 is localized in the nucleus and is involved in tumor cell proliferation and invasion. In particular, it plays a role in promoting tumor formation

by regulating the activity of miRNAs through RNA editing [46]. However, the antibodies used in this study are labeled p150 and p110, so it is impossible to distinguish between them. Both nuclear and cytoplasmic ADAR1 in cancer cells labeled by immunostaining showed a positive correlation with the staining of ADAR1 in macrophages, and the results showed that a strong staining intensity was associated with a poor prognosis. On the other hand, since macrophages are small cells, it is not possible to distinguish between nuclear and cytoplasmic ADAR1 using immunostaining, so we evaluated the entire cell. In CRC, both nuclear and cytoplasmic ADAR1 in cancer cells showed a strong correlation with ADAR1-high macrophages, so we did not clearly distinguish between p150 and p110.

Additionally, the reason we used a xenograft model of HCT116 and dTHP-1 mixed in an animal model is as follows. One of the objectives of our study was to collect macrophages from human peripheral blood and combine them with HCT116 cells. However, macrophages collected from peripheral blood mononuclear cells generally have a very short lifespan of a few days to a week. For this reason, it is challenging to utilize peripheral blood-derived macrophages in xenograft model experiments that last longer than 20 days. For this reason, dTHP-1 is frequently used in previous studies to perform co-culture experiments involving tumor cells and macrophages [47]. We would like to consider this as a topic for future study.

Conclusions

The findings of this study revealed that ADAR1-high TAMs are induced by stimulation from CRC cells and induce drug resistance. The combination treatment of oxaliplatin and JAK inhibitors based on the drug repositioning perspective suggests that it may be possible to unlock oxaliplatin resistance in CRC. Therapy that inhibits RNA-editing in cancer cells and macrophages is expected to be a promising approach to managing the entire tumor microenvironment.

Abbreviations

ADAR	Adenosine deaminase acting on RNA
ATCC	American Type Culture Collection
AZIN1	Antizyme inhibitor 1
CCL	Cancer Cell Line Encyclopedia
CRC	Colorectal cancer
EV	Extracellular vesicle
IF	Immunofluorescence
IFN	Interferon
IHC	Immunohistochemistry
IRS	Immunoreactive score
KEGG	Kyoto Encyclopedia of Genes and Genomes
PMA	Phorbol 12-Myristate 13-acetate
RECIST	Response Evaluation Criteria in Solid Tumors
RESS q-PCR	RNA-editing site-specific quantitative PCR
TAM	Tumor-associated macrophages

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12943-025-02312-y>.

Supplementary Material 1.

Supplementary Material 2.

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Authors' contributions

Conceived and designed experiments: HU, KS, T. Fuji, K. Yasui, HY, HM, KN, HT, YU, AG, T. Fujiwara. Conceived and designed experiments: HU, KS, T. Fuji, K. Yasui, HY, HM, KN, HT, YU, AG, T. Fujiwara. Performed experiments: HU, KS, TT, KM, T. Fuji, MK. Contributed reagents, materials, and other analytical tools: HU, KS, YK, K. Yoshida, ST, Y. Matsumi, Y. Mori, SY, HK, T. Fuji, FT. Wrote the manuscript: HU, KS, TT, KM, T. Fuji.

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Data Availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Written informed consent was obtained from each patient, and the institutional review board (The Ethics Committee of the Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences and Okayama University Hospital) approved the study (1903–037).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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