

Involvement of MAF1 homolog, negative regulator of RNA polymerase III in colorectal cancer progression

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Abstract. Polymerase (Pol) III-dependent transcription controls the abundance of transfer RNAs, 5S ribosomal RNA and small non-coding RNAs within cells, and is known to serve an essential role in the maintenance of intracellular homeostasis. However, its contribution to cancer progression has not been extensively explored. The present study demonstrated that the evolutionarily conserved MAF1 homolog, negative regulator of RNA Pol III (MAF1) may be closely associated with malignant potential and poor prognosis in colorectal cancer (CRC). Notably, immunohistochemical analysis of 146 CRC surgical specimens revealed that high expression levels of MAF1 were associated with advanced tumor depth, lymph node metastasis, distant metastasis and poor prognosis. *In vitro* loss-of-function assays revealed that MAF1 knockdown suppressed chemoresistance and migration of CRC cancer cells. Furthermore, detailed analysis of an independent CRC dataset (n=615) demonstrated that the prognostic impact of MAF1 gene expression was particularly marked in microsatellite instability (MSI)-positive patients, who benefit from immune checkpoint blockade. High expression levels of MAF1 were revealed to be an independent prognostic indicator in MSI-positive CRC. These findings suggested that MAF1 may have an essential role in CRC progression, particularly in MSI-positive cases.

Introduction

Colorectal cancer (CRC) is the third most common type of cancer in men and the second most common in women worldwide; every year, ~1.4 million new cases of CRC are diagnosed

and it is responsible for ~700,000 cases of mortality (1,2). Although screening and multidisciplinary treatment has improved therapeutic outcomes in several countries, due to its high incidence, CRC remains a major healthcare challenge worldwide. Therefore, specific biomarkers for predicting treatment outcomes, as well as key target molecules responsible for cancer progression, need to be identified.

Transcriptional activity is frequently dysregulated in cancer, due to genomic and epigenetic alterations. Polymerase (Pol) II is responsible for the transcription of mRNAs, including oncogenes, and other Pol enzymes, including Pol I and III, are also involved in cancer progression via ribosomal RNA (rRNA) and transfer RNA (tRNA) biosynthesis (3,4). Although transcription of protein-coding genes is Pol II-dependent, oncogenes and tumor suppressors, including MYC, phosphoinositide 3-kinase and phosphatase and tensin homolog directly or indirectly activate or inhibit transcription by Pol I and III, thus resulting in altered rRNA production, which is required for rapid cell growth (5).

The present study focused on MAF1 homolog, negative regulator of RNA Pol III (MAF1). Although MAF1 has been demonstrated to inhibit Pol III-dependent transcription, a certain number of Pol II-dependent genes are also considered to be important targets of this gene (6,7). For example, previous studies demonstrated a conserved function of MAF1 in the maintenance of intracellular lipid pools, through regulation of fatty acid synthase and acetyl-coA carboxylase expression (8,9). These findings suggest that MAF1 may not only be involved in a cell growth through rRNA- and tRNA-mediated post-transcriptional regulation, but may also affect various biological and pathological processes, including malignant potential of cancer.

Previous studies have reported the tumor suppressive effect of MAF1 in certain solid malignancies (7,8,10). However, the clinical significance has not been investigated in CRC, which has a different genetic background and immune environment. The present study used surgical specimens and a large-scale, multi-layered open database to investigate the clinical significance of MAF1 in CRC. Furthermore, the association between MAF1 expression in cancer cells and tumor immunity, which is an important factor for predicting prognosis, was explored.

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Materials and methods

Clinical samples. Primary CRC specimens were collected from 146 patients who underwent surgery at the Department of Gastroenterological Surgery, Osaka University (Suita, Japan) between January 2011 and December 2012. All patients were clearly diagnosed with CRC, based on the clinicopathological criteria described by the Japanese Society for Cancer of the Colon and Rectum (11). None of the patients received preoperative chemotherapy or radiotherapy. Specimens were fixed in 10% buffered formalin overnight at room temperature, processed through graded ethanol solutions and embedded in paraffin. The follow-up periods ranged between 1 month and 7 years, with a mean of 4 years. All data, including age, sex, tumor size and depth, lymphatic invasion, lymph node metastasis, vascular invasion, liver metastasis, peritoneal dissemination, distant metastasis and histological grade were obtained from clinical and pathological records. The present study was approved by the Research Ethics Committee of Osaka University (approval ID: 08226) and written informed consent was obtained from all patients included in this study.

Cell lines and cell culture. The human CRC cell lines RKO, HT29, HCT-116, Colo205, SW480 and DLD-1 were purchased from the American Type Culture Collection (Manassas, VA, USA). These cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Waltham, MA, USA) at 37°C in a humidified incubator containing 5% CO₂.

RNA interference. Two types of MAF1-specific small interfering RNA (siRNA; Sigma-Aldrich; Merck KGaA) were used to knockdown MAF1 mRNA. MAF1 siRNAs, or the negative control siRNA, were transfected into RKO and HCT-116 cells, which were seeded at 2x10⁵ cells/well in a 2-ml volume in 6-well flat-bottomed microtiter plates, at a final concentration of 50 nM using Lipofectamine[®] 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). These cells were maintained at 37°C in a humidified incubator containing 5% CO₂ for 48 h, according to the manufacturer's protocol. The sequences of siRNAs against MAF1 were as follows: #1, CCACGCUCAAUGAGUCCUUTT; #2, GGCUCAAGCGAAUCGUCUUTT. The MISSION[®] siRNA Universal Negative Control (cat. no. SIC001; Sigma-Aldrich; Merck KGaA) was used as a negative control siRNA.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cultured cells using TRIzol[®] RNA Isolation Reagents (Invitrogen; Thermo Fisher Scientific, Inc.) 48 h post-transfection as previously described (12). RNA quality was confirmed (RNA concentration >0.5 µg/µl and optical density 260/280=1.8-2.0). cDNA was synthesized from 10 ng total RNA using the ReverTra[®] Ace qPCR RT Master Mix (Toyobo Life Science, Osaka, Japan), according to the manufacturer's protocol. qPCR was performed using a LightCycler[®] 2.0 system (Roche Applied Science, Penzberg, Germany) and LightCycler[®] FastStart DNA Master SYBR Green I (Roche Applied Science). The amplification conditions were as follows: Initial denaturation

at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 10 sec and extension at 72°C for 10 sec. Data were normalized to the expression of GAPDH, which was used as an internal control for each experiment. The following primers were used: MAF1, sense 5'-ctcacagctgactgtggagact-3', antisense 5'-aacatgtgtttgctgctcctg-3'; and GAPDH, sense 5'-agccacatcgctcagacac-3' and antisense 5'-gcccaatagaccaaatcc-3'.

Immunohistochemical staining. Protein expression levels of MAF1 were assessed by immunohistochemical staining of the 146 CRC specimens. The anti-MAF1 rabbit antibody (cat. no. HPA058548; Atlas Antibodies AB, Bromma, Sweden) and VECTASTAIN[®] Elite ABC Rabbit Immunoglobulin G kit (cat. no. PK-6101; Vector Laboratories, Inc., Burlingame, CA, USA) were used for immunohistochemical staining, according to the manufacturer's protocol. Specimens were fixed in 10% buffered formalin overnight at room temperature, processed through graded ethanol solutions and embedded in paraffin. Tissue sections (3.5 µm) were prepared from paraffin-embedded blocks. Following antigen retrieval in 10 mM citrate buffer (pH 6.0) at 115°C for 15 min using Decloaking Chamber[™] NxGen (Biocare Medical, Pacheco, CA, USA), and the slides were incubated overnight at 4°C with the primary antibody at 1:300 dilution, followed by incubation at room temperature for 30 min with the secondary antibody at 1:200 dilution. With reference to the Human Protein Atlas (image available from v18.proteinatlas.org/ENSG00000179632-MAF1/tissue/esophagus#img), an intensity score of 2 was assigned to nuclei stained as intensely as normal esophageal mucosa, whereas unstained nuclei were assigned a score of 0. Nuclei that exhibited weaker staining than normal esophageal mucosa were assigned a score of 1. In the subsequent analysis, a score of 0 was defined as the MAF1-negative group, and a score of 1 or 2 was defined as the MAF1-positive group. Staining was reviewed by three independent pathologists without the knowledge of patient outcomes. The specimens were visualized on the light field of a confocal microscope BZ-X710 (Keyence Corporation, Osaka, Japan) and were analyzed using a BZ-X analyzer (v. 1.3.0.3; Keyence Corporation).

Scratch wound healing assay. Cells were seeded at a density of 5x10⁵ cells/well in 6-well plates and were grown to confluence under standard conditions. Briefly, a scratch was generated in the cell layer using a 200-µl pipette tip, and the cells were cultured under standard conditions in DMEM supplemented with only 1% FBS to prevent proliferation. Plates were washed with DMEM supplemented with 1% FBS to remove non-adherent cells prior to image capture. Images were captured at 0, 24 and 48 h after scratch generation using a confocal microscope BZ-X710 (Keyence Corporation) and were analyzed using a BZ-X analyzer (v. 1.3.0.3; Keyence Corporation). Cell migration was evaluated by measuring the average distance between the wound edges at 10 random areas.

Chemosensitivity assay. Cells were seeded at a density of 4x10³ cells/well in 96-well plates and were precultured for 24 h. Subsequently, the cells were exposed to various concentrations of 5-fluorouracil (5-FU; Tokyo Chemical Industry Co., Ltd.,

Tokyo, Japan) and oxaliplatin (L-OHP; Yakult Honsha Co., Ltd., Tokyo, Japan) at 37°C in a humidified incubator containing 5% CO₂ for 72 h. The *in vitro* cytotoxic effects of 5-FU and L-OHP were evaluated using the Cell Counting kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), according to the manufacturer's protocol. The half maximal inhibitory concentration values were calculated from the viability data of CRC cells treated with each concentration of L-OHP. The results were analyzed using Bioconductor package 'drc' (<https://cran.r-project.org/web/packages/drc/index.html>) with default settings; this package uses non-linear regression models.

Western blot analysis. Total proteins were extracted from cultured cells using radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors (Thermo Fisher Scientific, Inc.) and concentration was determined using the bicinchoninic acid method. Briefly, 15 µg proteins were separated by 10% SDS-PAGE and were electrophoretically transferred onto polyvinylidene fluoride membranes (Merck KGaA, Darmstadt, Germany) at 300 mA for 60 min. After blocking with 3% skim milk at room temperature for 1 h, these membranes were incubated with primary antibodies against MAF1 (cat. no. HPA058548; Atlas Antibodies AB), poly (ADP-ribose) polymerase (PARP), cleaved PARP, caspase-3, cleaved caspase-3 (apoptosis antibody sampler kit; cat. no. 9915; Cell Signaling Technology, Inc., Danvers, MA, USA) and actin (cat. no. A2066; Sigma-Aldrich; Merck KGaA) at 4°C overnight. After incubating with secondary antibodies (1:100,000; cat. no. NA934; GE Healthcare, Little Chalfont, UK), the protein bands were detected using the Amersham Enhanced Chemiluminescence Prime Western Blotting Detection Reagent (GE Healthcare).

Data processing. Experiments were conducted in triplicate, and data are presented as the means ± standard deviation. The Cancer Genome Atlas (TCGA) mRNA expression data and clinical information were downloaded from the GDAC Firehose website (<http://gdac.broadinstitute.org>); the colorectal adenocarcinoma data set (COADREAD) (n=615) was used. Regulatory T cell (Treg) infiltration in each sample was calculated using CIBERSORT (<http://cibersort.stanford.edu>) (13,14). R programming language v3.5.0, JMP® Pro 13.2.1 (SAS Institute Inc., Cary, NC, USA) and Microsoft® Excel® Version 14.7.1 (Microsoft Corporation, Redmond, WA, USA) were used for analysis. Overall survival (OS) rates and relapse-free survival (RFS) rates were calculated according to the Kaplan-Meier method and were measured from the day of surgery. Differences between groups were estimated using χ^2 test, Student's t-test, one-way analysis of variance followed by post hoc Dunnett test, or the log-rank test. For univariate and multivariate analyses of clinicopathological factors, variables with P<0.05 in the univariate analysis were used in a subsequent multivariate analysis based on the Cox proportional hazards regression model. P<0.05 was considered to indicate a statistically significant difference.

Results

High MAF1 expression is associated with advanced clinicopathological factors and poor prognosis. Of the

Table I. MAF1 expression and clinicopathological factors of patients with colorectal cancer.

Factor	Positive (n=97) Number (%)	Negative (n=49) Number (%)	P-value
Age (mean ± SD)	63.8±1.35	67.8±1.56	0.0544
Sex			
Male	60 (61.9)	32 (65.3)	0.6827
Female	37 (38.1)	17 (34.7)	
Histological grade			
tub1, tub2, pap	87 (89.7)	45 (91.8)	0.6738
por, muc	10 (10.3)	4 (8.2)	
Depth of tumor invasion			
Tis, T1, T2	36 (37.1)	31 (63.3)	0.0027 ^a
T3, T4	61 (62.9)	18 (36.7)	
Lymph node metastasis			
Absent	61 (62.9)	44 (89.8)	0.0003 ^a
Present	36 (37.1)	5 (10.2)	
Lymphatic invasion			
Absent	32 (33.0)	23 (47.0)	0.2139
Present	64 (66.0)	25 (51.0)	
No data	1 (1.0)	1 (2.0)	
Venous invasion			
Absent	67 (69.1)	39 (79.6)	0.2890
Present	29 (29.9)	9 (18.4)	
No data	1 (1.0)	1 (2.0)	
Distant metastasis			
Absent	83 (85.6)	47 (95.9)	0.0417 ^a
Present	14 (14.4)	2 (4.1)	
Stage			
0, I, II	57 (58.8)	44 (89.8)	0.0011 ^a
IIIa, IIIb, IV	40 (41.2)	5 (10.2)	

^aP<0.05. MAF1, MAF1 homolog, negative regulator of RNA polymerase III; muc, mucinous adenocarcinoma; pap, papillary adenocarcinoma; por, poorly differentiated adenocarcinoma; SD, standard deviation; Tis, tumor *in situ*; tub1, well differentiated tubular adenocarcinoma; tub2, moderately differentiated tubular adenocarcinoma.

146 CRC cases, 97 cases were classified as MAF1-positive (52 cases with strong staining and 45 cases with weak staining), whereas 49 cases were classified as the MAF1-negative (Fig. 1A). A clinicopathological analysis demonstrated that high MAF1 expression was associated with tumor malignancy, including tumor depth, lymph node metastasis, distant metastasis and a poorer cancer stage (Table I). Kaplan-Meier curves for OS (n=146) and RFS (n=130) revealed that the MAF1-positive group exhibited a significantly poorer prognosis (P=0.0123) and higher relapse rate (P=0.0132) compared with the MAF1-negative group (Fig. 1B and C). Univariate analysis indicated that tumor depth, lymph node metastasis, lymphatic invasion, venous invasion and MAF1 expression were prognostic factors for

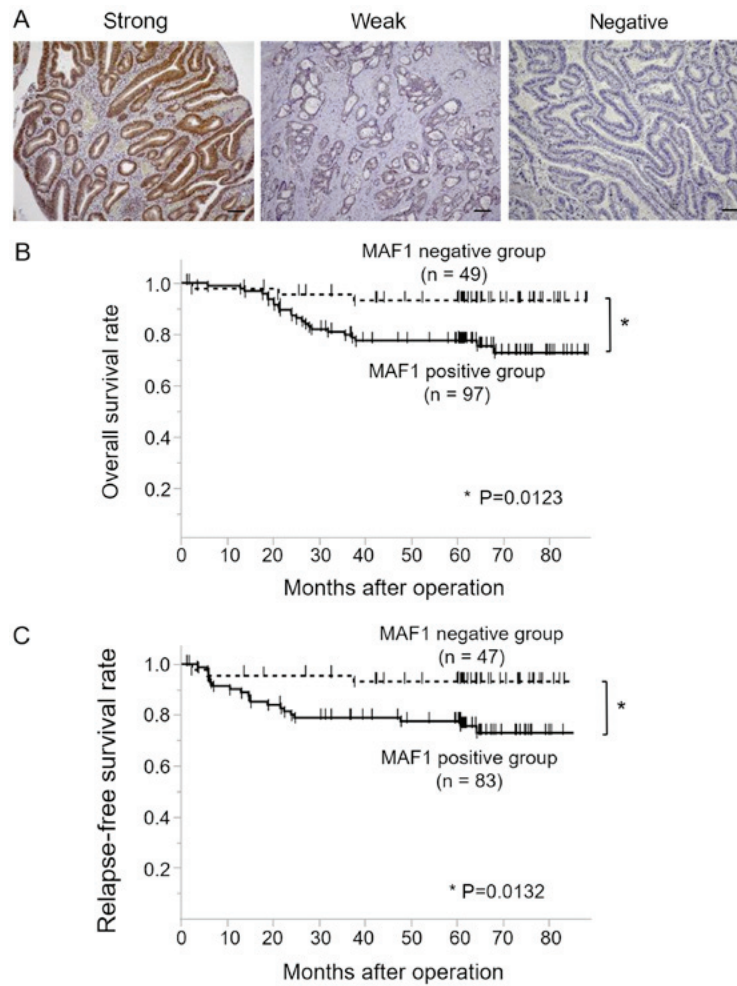


Figure 1. Immunohistochemical analysis of MAF1 expression and its prognostic impact in CRC. (A) Representative cases of immunohistochemical staining with anti-MAF1 antibody. Examples of strong intensity, weak intensity and negative staining are presented. Scale bar, 100 μ m. Negative staining was defined as the MAF1-negative group, whereas strong and weak staining was defined as the MAF1-positive group. Kaplan-Meier curves for (B) overall survival and (C) recurrence-free survival, according to MAF1 expression status in patients with CRC. CRC, colorectal cancer; MAF1, MAF1 homolog, negative regulator of RNA polymerase III.

Table II. Univariate and multivariate analyses of overall survival in patients with colorectal cancer (Cox regression model).

Factors	Univariate analysis			Multivariate analysis		
	RR	95% CI	P-value	RR	95% CI	P-value
Age ($\leq 65 / > 65$ years)	0.59	0.26-1.28	0.1855	-	-	-
Sex (male/female)	1.35	0.60-3.28	0.4769	-	-	-
Histology grade (muc, por/tub1, tub2, pap)	2.65	0.88-6.54	0.0778	-	-	-
Depth of tumor invasion (T3, T4/Tis, T1, T2)	11.8	3.50-73.4	<0.0001 ^a	7.76	1.95-52.7	0.0021 ^a
Lymph node metastasis (positive/negative)	5.28	2.42-12.1	<0.0001 ^a	2.84	1.10-8.30	0.0303 ^a
Lymphatic invasion (positive/negative)	3.34	1.27-11.5	0.0123 ^a	0.49	0.13-2.10	0.3138
Venous invasion (positive/negative)	5.13	2.32-11.8	<0.0001 ^a	2.46	1.05-6.09	0.0389 ^a
MAF1 expression (positive/negative)	4.12	1.43-17.4	0.0062 ^a	1.94	0.64-8.43	0.2666

^aP<0.05. CI, confidence interval; MAF1, MAF1 homolog, negative regulator of RNA polymerase III; muc, mucinous adenocarcinoma; pap, papillary adenocarcinoma; por, poorly differentiated adenocarcinoma; RR, relative risk; Tis, tumor *in situ*; tub1, well differentiated tubular adenocarcinoma; tub2, moderately differentiated tubular adenocarcinoma.

overall survival, whereas multivariate analysis revealed that tumor depth, lymph node metastasis and venous invasion were independent prognostic factors (Table II).

MAF1 expression is associated with the malignant potential of CRC cells. To elucidate how MAF1 contributes to the malignant potential of CRC, *in vitro* loss-of-function assays were

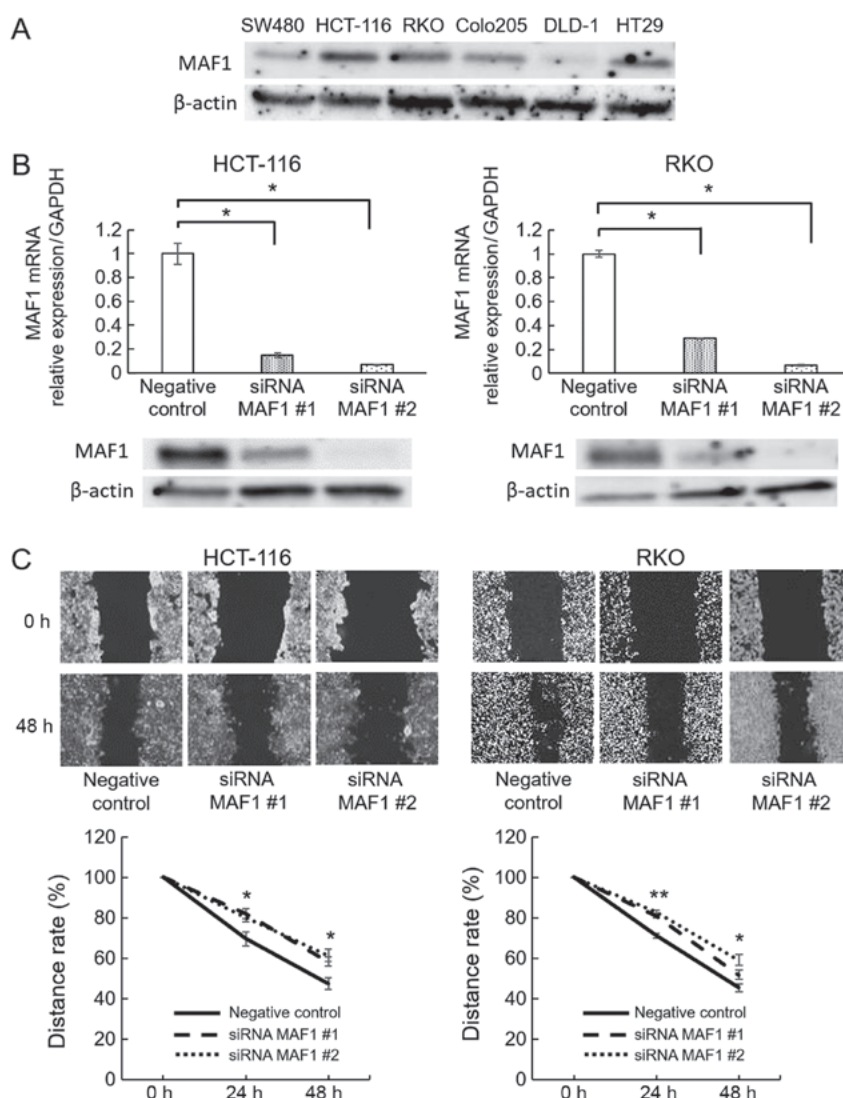


Figure 2. MAF1 is involved in the migratory ability of CRC cells. (A) Western blotting of MAF1 in CRC cell lines. (B) MAF1 expression levels in HCT-116 and RKO cells transfected with negative control siRNA or siRNAs against MAF1. mRNA expression levels were detected by reverse transcription-quantitative polymerase chain reaction and were normalized to GAPDH. * $P < 0.001$ (upper panel). Protein expression levels were detected by western blotting (lower panel). (C) Representative images of the scratch wound healing assay post-transfection of HCT-116 and RKO cells with negative control siRNA or siRNAs against MAF1 (upper panel; magnification, $\times 100$). Distance between wound edges at the indicated time points (normalized to distance at 0 h); the average of 10 different areas was obtained (lower panel). * $P < 0.05$, ** $P < 0.001$ vs. negative control. CRC, colorectal cancer; MAF1, MAF1 homolog, negative regulator of RNA polymerase III; siRNA, small interfering RNA.

performed using RNA interference. HCT-116 and RKO CRC cell lines were used for MAF1 knockdown experiments, since they exhibited relatively high expression levels of MAF1 in the CRC cell lines investigated (Fig. 2A). Knockdown efficiency was confirmed using RT-qPCR and western blotting (Fig. 2B). Subsequently, MAF1 knockdown significantly inhibited the migratory ability of HCT-116 and RKO cells (Fig. 2C), whereas proliferation rate was not affected (data not shown). To further investigate the involvement of MAF1 in CRC progression, the association between drug resistance of cancer cells and MAF1 expression was explored. The results from the chemosensitivity assay revealed that knockdown of MAF1 significantly improved chemosensitivity to L-OHP, which is one of the standard chemotherapy drugs used to treat CRC (Fig. 3A), whereas chemosensitivity to 5-FU was not affected (data not shown). Western blotting was performed to confirm the enhanced induction of apoptosis in MAF1-knockdown

cells treated with L-OHP. Knockdown of MAF1 markedly enhanced the expression of cleaved PARP and caspase-3 in L-OHP-treated cells, thus demonstrating that MAF1 may be critically involved in the avoidance of CRC cell apoptosis following exposure to cytotoxic agents (Fig. 3B).

High MAF1 expression is associated with poor prognosis in CRC with microsatellite instability (MSI). To further evaluate the clinical significance and prognostic impact of MAF1 expression in CRC, the present study analyzed a large-scale CRC dataset from TCGA ($n=615$), which consisted of 87 MSI-positive cases and 528 non-MSI cases. Since Pol III activity is closely associated with immune response in various cell types (15,16), this study focused on MAF1 expression and MSI status, which strongly affects activation of tumor immunity. 'MSI-high' samples were considered MSI-positive, whereas all other samples were categorized in the non-MSI

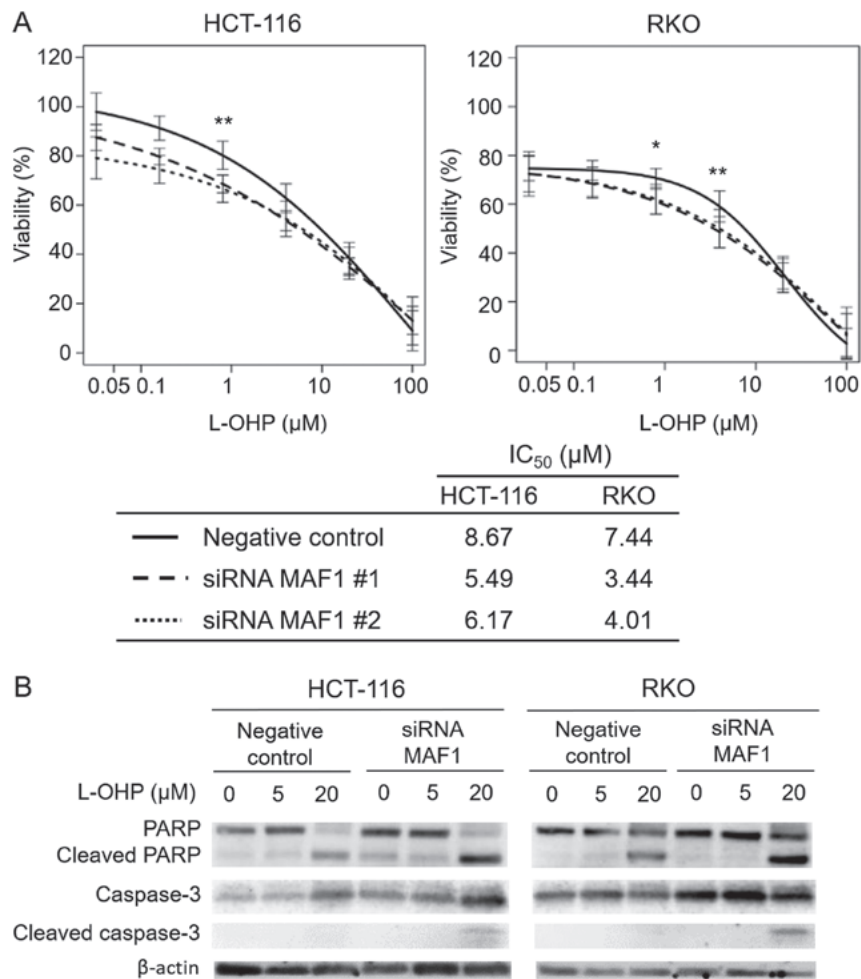


Figure 3. MAF1 inhibition sensitizes colorectal cancer cells to the chemotherapeutic agent L-OHP. (A) Dose-response curves for viability of HCT-116 and RKO cells. Each bar represents the means \pm standard error of the mean of samples measured in triplicate. * $P < 0.05$, ** $P < 0.001$ vs. negative control. IC₅₀ values in each cell line are shown. (B) Western blotting images of PARP and caspase-3 cleavage following L-OHP treatment with or without MAF1 knockdown. HCT-116 and RKO cells were treated with 5 and 20 μM oxaliplatin for 72 h post-transfection with negative control siRNA or siRNA against MAF1. IC₅₀, half maximal inhibitory concentration; L-OHP, oxaliplatin; MAF1, MAF1 homolog, negative regulator of RNA polymerase III; PARP, poly (ADP-ribose) polymerase; siRNA, small interfering RNA.

Table III. Univariate and multivariate analyses of overall survival in MSI cases using The Cancer Genome Atlas dataset (Cox regression model).

Factor	Univariate analysis			Multivariate analysis		
	RR	95% CI	P-value	RR	95% CI	P-value
Age ($\leq 65 / > 65$ years)	0.33	0.05-1.19	0.0962	-	-	-
Sex (male/female)	0.24	0.06-0.76	0.0131 ^a	0.21	0.05-0.69	0.0079 ^a
Depth of tumor invasion (T3, T4/T1, T2)	3.21	0.65-58.2	0.1797	-	-	-
Lymph node metastasis (negative/positive)	0.82	0.30-2.61	0.7124	-	-	-
MAF1 expression (low/high)	0.25	0.07-0.70	0.0075 ^a	0.22	0.06-0.64	0.0046 ^a

^a $P < 0.05$. CI, confidence interval; MAF1, MAF1 homolog, negative regulator of RNA polymerase III; RR, relative risk; Tis, tumor *in situ*.

group; this is because of the differences in clinical and immunological behaviors between MSI-high tumors and other tumors (17,18). In the subgroup analysis using MSI status information, the prognostic impact of MAF1 was significantly higher in MSI-positive cases ($P = 0.00845$) compared with all

cases ($P = 0.0146$) or non-MSI cases ($P = 0.157$) (Fig. 4A-C). Multivariate analysis of MSI-positive cases ($n = 87$) indicated that high MAF1 expression was an independent prognostic factor for overall survival (Table III). To further investigate the association between MAF1 expression and the immune

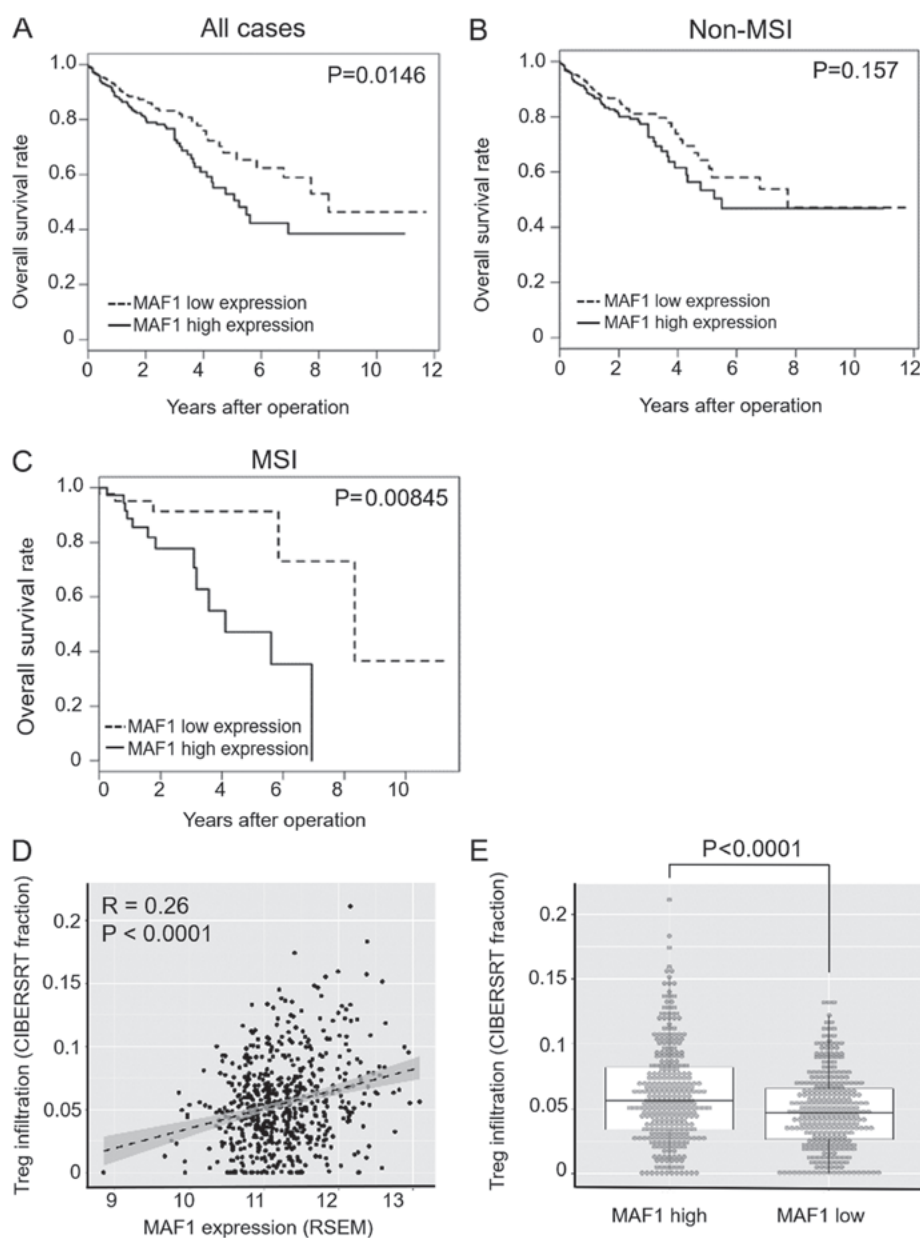


Figure 4. Prognostic impact of MAF1 expression in MSI tumors and involvement of MAF1 in the immune microenvironment. Kaplan-Meier curves of overall survival in (A) all colorectal cancer cases (n=615), (B) non-MSI cases (n=528) and (C) MSI-positive cases (n=87). (D) Correlation between MAF1 expression and Treg infiltration in The Cancer Genome Atlas datasets. Dashed line and gray area indicate linear regression line and 95% confidence interval, respectively (correlation coefficient, $R=0.26$, $P<0.0001$). (E) Treg infiltration fraction was calculated using CIBERSORT; Treg infiltration fraction in MAF1 high and low expression groups is shown. Median of the expression in all cases was used as a cut-off level. MAF1, MAF1 homolog, negative regulator of RNA polymerase III; MSI, microsatellite instability; Treg, regulatory T cell.

microenvironment, bioinformatics analysis was performed using CIBERSORT (13,14). CIBERSORT is a computational program used to predict the relative contribution of each immune cell type in a mixed cell population using transcriptome data (13,14). A mild correlation between MAF1 expression and Treg infiltration was detected (Fig. 4D), and the extent of Treg infiltration was significantly higher in the MAF1 high expression group in the TCGA dataset ($P<0.0001$; Fig. 4E).

Discussion

The present used two independent datasets and demonstrated that high MAF1 expression may be closely associated with

cancer progression in patients with CRC. The results of clinicopathological analysis revealed that high MAF1 expression was highly associated not only with tumor depth, but also with lymph node and distant metastasis, thus suggesting that MAF1 may contribute to metastatic ability. In addition, *in vitro* analyses revealed that MAF1 inhibition significantly suppressed the migratory ability of CRC cells. MAF1 knockdown also rendered cancer cells more sensitive to a chemotherapeutic agent. In the present analysis, MAF1 was significantly highly expressed in the patients with stage III and IV cancer; these patients have been reported to benefit from standard chemotherapy (19). Therefore, the MAF1 gene may be a useful biomarker, as well as therapeutic target, for patients receiving chemotherapy. However, further *in vivo* investigation

is required to fully confirm the improvement of chemoresistance to L-OHP by knockdown of MAF1 expression.

High MAF1 expression was not considered an independent prognostic indicator in all patients with CRC, whereas analysis of an MSI-positive population clearly demonstrated that MAF1 was a significant independent prognostic indicator. These findings suggested that the prognostic value of MAF1 expression may be particularly important in MSI-positive patients.

Although MAF1 expression has been reported to suppress cancer proliferation through negative regulation of Pol III-mediated transcription in some cell lines, the malignant potential of MAF1, including its effects on chemoresistance, have not been sufficiently explored. Cellular stress, which can cause growth arrest, also induces cell dormancy, leading to stress tolerance and cell survival, depending on cellular context (20,21). Notably, MAF1 has been reported to be required for maintaining a cell dormancy-like state and subsequent cell survival under nutrient starvation in *Plasmodium falciparum* (22). Furthermore, previous reports have revealed that MAF1 can directly or indirectly regulate Pol II-dependent genes (7,8,23), indicating that MAF1 may control various cellular functions, including the malignant potential of cancer cells. However, since this study did not identify direct targets of the MAF1 gene that regulate cell mobility and survival, further exploration is required to determine the direct involvement of MAF1 in the metastatic process of CRC.

The present results obtained from clinical samples indicated an oncogenic role for the MAF1 gene in CRC; however, other reports have demonstrated a tumor suppressive role of MAF1 in other malignancies, with the exception of CRC (8,10). To explain this discrepancy, this study focused on the immune microenvironment, which differs markedly among tissues. Growing evidence has suggested that both tumor antigenicity and the immune environment have a marked influence on the prognosis of patients with cancer (24,25). In addition, because Pol III activity is known to be closely associated with the immune response in various cell types (15,16), the present study considered the possibility that MAF1 may serve an important role in the immune microenvironment. Immune checkpoint inhibitors are increasingly being used in the clinic, and have been reported to be very effective for the treatment of MSI-high tumors, regardless of histology (18). This is because MSI tumors activate the immune system through producing a large number of neoantigens (18,26-29). Notably, the present subgroup analysis using a CRC TCGA database revealed that the prognostic impact of MAF1 was more evident in MSI-high CRC, thus suggesting that MAF1 may exert its malignant potential more powerfully under immune-activated conditions. In this context, transcriptome-based gene enrichment analysis demonstrated that Treg infiltration was more abundant in tumors with high expression levels of MAF1. Tregs are known to suppress the immune response against tumor antigens in several types of tissue (30), thus indicating a close association between MAF1 expression in cancer and immune suppression in CRC tissues. However, further investigation is required to fully understand the effects of MAF1 expression on tumor immunity.

In conclusion, the present study revealed that MAF1 expression may be considered a useful prognostic indicator in patients with CRC, particularly in MSI-positive cases. These results may help to understand the complex gene regulatory

network, including both Pol II and III-dependent transcription, and may provide novel medical information that may lead to breakthroughs in the treatment of CRC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contribution

KH initiated this project. KH, NN, NM, HT, NH, TH, CM and TM designed experiments and wrote the manuscript. KH and NN performed *in vitro* experiments and bioinformatics analysis. CM, TM, YD and MM provided clinical samples and designed the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of Osaka University (approval ID: 08226) and written informed consent was obtained from all patients included in this study.

Patient consent for publication

Consent for publication was obtained from all patients included in this study.

Competing interests

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