BAF57 Is a Potential Determinant of Colorectal Cancer Malignancy

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Abstract. *Background/Aim: The role of brahma-related gene 1 (BRG1)-associated factor 57 (BAF57), a transcription factor, has been determined in prostate, breast, and ovarian cancer. However, the relationship between BAF57 and colorectal cancer (CRC) is obscure. Thus, we examined the functional correlation between BAF57 expression and oncological malignancy in CRC in vitro. Materials and Methods: BAF57 expression in WiDr and HT29 CRC cell lines and clinical specimens from CRC patients was analysed by western blotting and/or RT-PCR. BAF57 expression was down-regulated in WiDr cells through siRNA transfection. An invasion assay was also performed to assess malignancy. Results: BAF57 was expressed in both human CRC cell lines. Overall survival and recurrence-free survival rates were significantly reduced in high BAF57-expressing specimens. BAF57 expression was an independent predictive factor for long-term survival. Conclusion: BAF57 correlates with oncological malignancy and may be a novel therapeutic target in CRC.*

Colorectal cancer (CRC) affects more than one million people and causes more than half a million deaths annually worldwide (1). Approximately 25% of patients with CRC present with metastatic disease with 40%-50% of newly diagnosed patients ultimately developing metastasis. Systemic chemotherapy for treating CRC has progressed remarkably in recent years. With the development of the anti-vascular

endothelial growth factor (VEGF) antibody bevacizumab and anti-epidermal growth factor receptor (EGFR) antibodies cetuximab and panitumumab, which function as molecular-targeted therapeutic agents, the survival of patients with advanced CRC has improved significantly (2-4). However, resistance to systemic chemotherapy remains an important clinical problem. In fact, several studies have reported resistance to molecular-targeted therapeutic agents (5-7). Miroddi *et al.* reported that anti-EGFR antibody may promote thromboembolism by extending the uncovering of endothelial structures caused by other co-administered agents (6). Therefore, it is important to identify new clinical and practical biomarkers for use in CRC.

Brahma-related gene 1 (BRG1)-associated factor 57 (BAF57) is a core subunit of the mammalian multimeric switching-defective/sucrose nonfermenting (SWI/SNF) complex that can alter DNA-nucleosome topology (8). Energy from ATP hydrolysis changes the location or conformation of nucleosomes in the SWI/SNF complex (9). This functions to induce chromatin remodelling, accompanied by transcriptional activation and gene repression. BAF57 mediates direct interactions with hormone receptors (oestrogen and androgen) to regulate transcriptional activity (10-12). Accordingly, Yamaguchi *et al.* have reported that BAF57 is related to cell growth and sensitivity to anticancer agents in ovarian cancer (11). Furthermore, Kagami *et al.* reported that inhibition of BAF57 activity may be a target for endometrial cancer therapy (12). Additionally, BAF57 expression is reportedly associated with metastasis in prostate cancer (13). Specifically, cell differentiation and migration may be promoted by BAF57, resulting in the induction of metastasis Therefore, BAF57 has the potential to play an important role in the control of hormone-dependent proliferation of hormone-sensitive cancer cells. Even in CRC, the lack of oestrogen receptor β (ERβ) is associated with advanced stages of disease and is an independent factor associated with poor survival of patients

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(14). While it has been suggested that there is a relationship between CRC and hormones, there are currently no reports on the potential function and role of BAF57 in CRC.

Based on the above noted findings, we hypothesized that BAF57 has clinical significance and is a factor associated with oncogenesis and malignancy. In this study, we evaluated the expression of BAF57 in CRC cell lines and investigated the *in vitro* functional correlation between BAF57 expression and CRC malignancy.

Materials and Methods

Cell lines and culture conditions. The human CRC-derived cell lines WiDr and HT29 were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich Co., St. Louis, MO, USA) supplemented with 10% foetal bovine serum (CSL Ltd., Melbourne, Australia) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). The cells were incubated at 37˚C.

Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR). To confirm the expression of BAF57 in human CRC cells, we first measured BAF57 mRNA levels. Total RNA was extracted from the cells and reverse transcribed into complementary DNA (cDNA) as previously described (15). Real-time PCR analysis was performed to evaluate the gene expression using a ViiA 7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and Fast SYBR Green Master Mix (Thermo Fischer Scientific) according to the manufacturer's instructions. Reactions were carried out using primers specific for human BAF57 (hBAF57). Jurkat cells constitutively expressing hBAF57 were used as a positive control and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal loading control. The primer sequences were as follows: *BAF57* forward primer, 5'-GTAGGGCATCAGCGGCTTAT-3'; *BAF57* reverse primer, 5'-CTCCTGCAACACAAATGCCC-3'; and *GAPDH* forward primer, 5'-ATCATCCCTGCCTCTACTGG-3'; and *GAPDH* reverse primer, 5'-TTTCTAGACGGCAGGTCAGGT-3'). *BAF57* expression levels in the human CRC cell lines were normalized to those in the Jurkat cells. We used the comparative cycle time $(2^{-\Delta\Delta CT})$ method to quantify gene expression. The mRNA expression values were set as log2 fold-change of the mean $-\Delta^{CT}$ difference. Each experiment was performed in duplicate.

Western blot analysis of BAF57 expression. Total cell lysates were extracted using lysis buffer as previously reported (16). The protein concentration in the cell lysates was determined using a Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). An aliquot of each lysate containing 25 μg of protein was resolved in Readygela J (Bio-Rad Laboratories) and transferred to Immuno-Blot™ polyvinylidene fluoride membranes (Bio-Rad Laboratories). The membranes were blocked for 1 h at room temperature in phosphate-buffered saline (Sigma-Aldrich) containing 5% non-fat milk and then incubated with anti-BAF57 (1:500; goat monoclonal; Santa Cruz Biotechnology, Dallas, TX, USA) and anti-β-actin (1:2,500; mouse monoclonal; BD Biosciences, San Jose, CA, USA) primary antibodies for 24 h at 4˚C. The membranes were then washed and incubated for 10 min with a horseradish peroxidase-conjugated anti-goat IgG secondary antibody (1:5,000; Promega Corp., Fitchburg, WI, USA). The targeted proteins were labelled with

Luminata Forte Western HRP Substrate (Merck Millipore Co., Darmstadt, Germany) according to the manufacturer's instructions. The bound complexes were immediately detected using a FluorChem FC2 Imaging System (Alpha Innotech, San Leandro, CA, USA). The staining density of the BAF57 band was determined by densitometry using AlphaView software (ProteinSimple, San Jose, CA, USA) and normalized against the staining density of the β-actin band. Each analysis was performed in triplicate.

Invasion assays. Invasion assays were performed to evaluate the malignancy of human CRC cell lines WiDr and HT29 according to a previously described method (17). First, the Transwell (Corning Incorporated) insert membranes were coated with 100 μl of Matrigel (200 μg/ml, cat. no. 354234; Corning Life Sciences, Tewksbury, MA, USA) and dried at 37˚C for 12 h. Then, 250 μl of culture medium containing 10% foetal bovine serum was added to the insert membranes and allowed to sit undisturbed for 90 min to equilibrate the Matrigel. Prior to completion of the equilibration period, the culture cells were adjusted to a concentration of 4×10^4 cells/ml. Once the equilibration was complete, the entire culture medium was aspirated, and sterile forceps were used to transfer the insert membranes to the outer Transwell chambers that had been pre-filled with 750 μl of culture medium. Then, 250 μl of the culture cells $(4\times10^4/\mu l)$ were added to the chamber and the cells incubated at 37˚C in a 5% CO_2 humidified incubator for 48 h.

After incubation, paraformaldehyde (PFA) at a final concentration of 4% was added to the culture medium on bottom of each Transwell insert for 15 min to fix the cells. The culture medium and 4% PFA were removed by gentle aspiration and the membranes dried at room temperature. After drying, the membranes were removed from the chambers using a scalpel and placed onto microscope slides. A fluorescence microscope was used to count the number of invading cells in five random microscopic fields at 400× magnification for each membrane.

BAF57 small interfering RNA (siRNA). WiDr cells were transfected with BAF57 siRNA (Santa Cruz Biotechnology, Inc.) to interfere with BAF57 mRNA or scrambled siRNA (Santa Cruz Biotechnology, Inc.) as a negative control using the methods described previously (18). The sequence of the scrambled siRNA duplex was 5'-ATAAGCTG TGGTACCATCTTCCAGC-3'. Lipofectamine 2000 (Invitrogen-Life Technologies, Inc., Carlsbad, CA, USA) was used for transfecting the siRNAs according to the manufacturer's instructions. Briefly, 1×10^6 WiDr cells were plated onto 6 cm plates and incubated for 24 h. The cells were then treated for 20 min with various concentrations of siRNA mixed with 10 μl of Lipofectamine 2000 in serum-free OptiMEM medium. The cells were then gently mixed and incubated for a further 20 min. The transfectants were evaluated for protein expression by western blotting and for changes in invasion ability.

Clinical specimens. BAF57 expression levels were analysed in tissue specimens resected from patients with CRC that were treated at our hospital from September 2015 to November 2016. Eligible patients were those meeting the following inclusion criteria: the main tumour located in the colon or rectum that was histologically confirmed as adenocarcinoma, mucinous adenocarcinoma, or signet ring cell carcinoma; pathologically diagnosed as T2-T4, N0-N3, M0 lesions without invasion of other organs based on preoperative abdominal computerized tomography scans or magnetic resonance imaging; no multiple tumours observed by endoscopy or
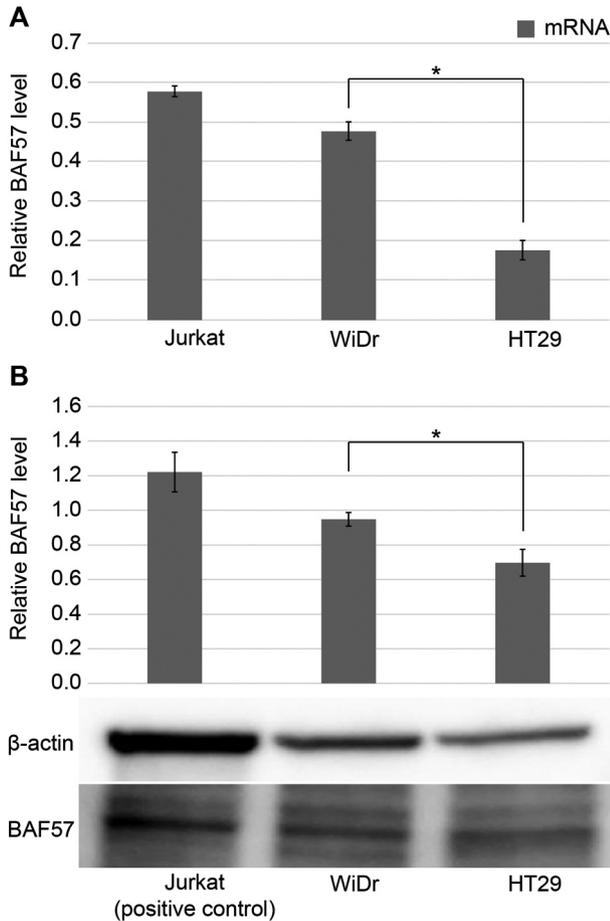
Figure 1. *Expression of BAF57 in human colorectal cancer cells. (A) BAF57 mRNA levels of two colorectal cancer cell lines (WiDr and HT29) were determined by real-time RT-PCR. Jurkat cells were used as a positive control. BAF57 mRNA levels in WiDr cells are significantly higher than those in HT29 cells. Error bars, SD. *p<0.05 by Mann-Whitney U-test. (B) Western blots and graphs of densitometric analysis show significantly higher BAF57 protein levels in WiDr cells compared to that in HT29 cells. Error bars, SD. *p<0.05 by Mann-Whitney U-test.*

Figure 2. *Differences in malignant potential between human WiDr and HT29 colorectal cancer-derived cells. Invasion assays were performed to evaluate invasive ability (malignant potential) and the number of invading cells was counted for the two human colon cell lines in five random microscopic fields of each membrane using fluorescence microscopy. The invasion ability of WiDr cells was significantly higher than that of HT29 cells. Error bars, SD. *p=0.003 by Mann-Whitney U-test.*

Statistical analysis. Each value is expressed as the median±standard error without any notation. Statistical analysis was performed using the Mann-Whitney *U*-test, regression analysis, or Cox proportional hazard model using STATA software (version 12.0; StataCorp LP, College Station, TX, USA). Statistical significance was set at *p*<0.05.

Results

Expression of BAF57 in human CRC cell lines. We first attempted to confirm the expression of BAF57 in human CRC cells using the WiDr and HT29 cell lines. Relatively high levels of BAF57 mRNA were observed in WiDr based on real-time RT-PCR analysis (Figure 1A). Mean BAF57 mRNA levels in the WiDr and HT29 CRC cells and that in the positive control Jurkat cells relative to GAPDH mRNA levels were 0.48, 0.18, and 0.59, respectively. The mean BAF57 mRNA level in WiDr cells was significantly higher than that in HT29 cells (0.48 *vs*. 0.18; *p*<0.05). Western blot analysis was performed to evaluate BAF57 protein levels (Figure 1B). Consistent with the mRNA analysis, the mean BAF57 protein levels relative to β-actin protein levels in WiDr cells was significantly higher than that in HT29 cells (0.97 *vs*. 0.68; *p*<0.05).

Correlation between BAF57 expression and CRC malignancy. We performed Transwell invasion assays to evaluate the malignant potential of the human CRC cell lines (Figure 2). Based on the number of invading cells in five random microscopic fields of each Transwell membrane evaluated, the invasion ability of WiDr cells was significantly higher than that of HT29 cells (WiDr: 132 cells *vs*. HT29: 67 cells; *p*=0.003). These results were consistent with the BAF57 expression

preoperative image assessment; and no preoperative therapy, including chemotherapy or radiotherapy. All patients provided written informed consent for the publication of patient data. The study was performed in accordance with the principles of the Declaration of Helsinki, and approved by the institutional ethics committee of Keio University (approval number: 20150148).

The specimens were immediately stored at −80˚C after surgery and later processed for RNA extraction and preparation of cDNA as previously described (15). Real-time RT-PCR was performed using the method described above to measure BAF57 mRNA levels. Reactions were carried out as described above using primers specific for hBAF57. We statistically examined the relationship between BAF57 expression levels and CRC malignancy. The patients were divided into two groups based on their average BAF57 expression values, low BAF57 expressing and high BAF57 expressing. The two groups were then statistically compared relative to their clinicopathological backgrounds and long-term prognosis.
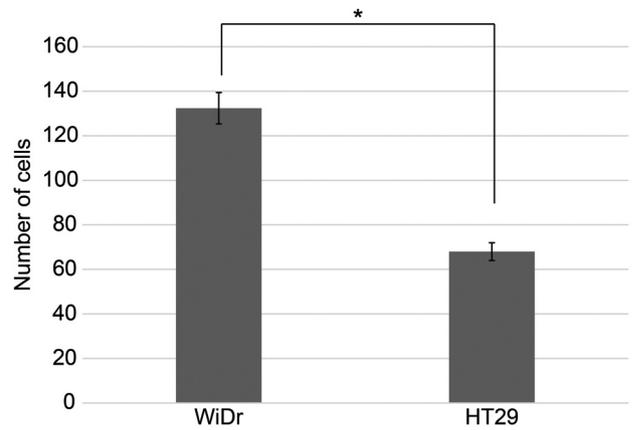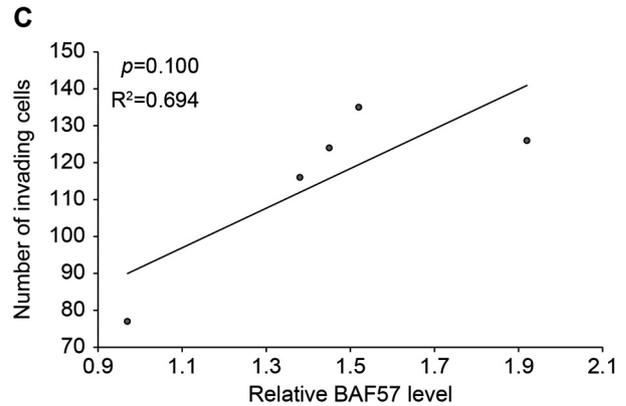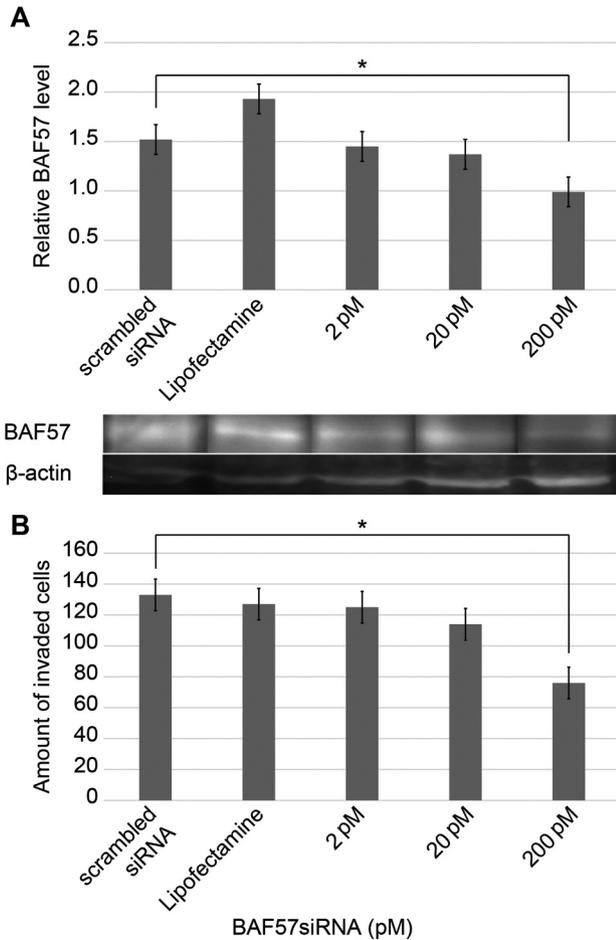
Figure 3. *Inhibition of BAF57 protein expression by siRNA suppression reduces the malignant potential of the colorectal cancer cell line WiDr. WiDr cells were transfected with BAF57 scrambled siRNA, lipofectamine 2000 alone, or various concentrations of BAF57 siRNA. (A) One day after treatment, total protein was exacted from cultures cells and BAF57 and β-actin protein levels evaluated by western blot analysis. Treatment of WiDr cells with BAF57 siRNA reduced BAF57 protein levels in a dose-dependent manner. Error bars, SD. *p<0.05 by Mann-Whitney U-test. (B) Colorectal cancer malignancy potential was evaluated by invasion assays. There was a significant correlation between cell invasion and BAF57 concentration. Error bars, SD. *p=0.031 by Mann-Whitney U test. (C) The correlation between BAF57 protein levels and the number of invading cells in BAF57 siRNA-treated WiDr cells was evaluated by regression analysis. There is a positive correlation between BAF57 expression levels and invasive ability (malignant potential) in colorectal cancer cells.*

results. A positive relationship was observed between malignant potential (invasion ability) and BAF57 expression.

We then examined whether changes in BAF57 expression affected the invasion ability by using an interference RNA method that was specific for BAF57. As shown in Figure 3A, transfection of WiDr cells with siRNA resulted in reduced BAF57 protein levels relative to β-actin protein levels in a dose-dependent manner (scrambled siRNA: 1.52 *vs*. BAF57 siRNA: 0.98, *p*<0.05). To determine whether BAF57down-regulation could mitigate the malignant potential of WiDr cells, invasion assays were performed following siRNA knockdown. As shown in Figure 3B, treatment of WiDr cells with BAF57 siRNA suppressed cell invasion based on the number of invading cells in five random microscopic fields of each Transwell membrane evaluated (scrambled siRNA: 134 cells *vs*. BAF57 siRNA: 77 cells; *p*<0.05). There was a trend toward a positive correlation between cell invasion and BAF57 protein level (Figure 3C), however, statistical significance was not reached (*p*=0.100).

Clinical specimens. Demographics of the patients enrolled in this study and the clinical characteristics of their tumour

specimens are summarized in Table I. A total of 35 male patients and 27 female patients with CRC were included in the study. The average BAF57 expression level was 0.309. The median age (range) and body mass index (range) of the patients was 68 (range=38-90) years and 21.8 (range=15.3-38.9) kg/m^2, respectively. Forty-one patients had primary tumours in the colon and 21 patients had tumours in the rectum. Twenty-seven patients received adjuvant chemotherapy after surgery. The median observation period was 46.8 months (range=11.2-57.2 months). Patients with higher BAF57 expression exhibited significantly lower overall survival rates (*p*<0.001) and lower recurrence-free survival rates (*p*=0.022) compared to those with lower BAF57 expression (Figure 4A and B).

Impact of the BAF57 value on overall survival based on univariate analysis using the Cox proportional hazards model is shown in Table II. Multivariate analysis using the Cox proportional hazards model adjusted for sex, tumour location, tumour size, pathological N factor, and adjuvant chemotherapy revealed that BAF57 expression was an independent risk factor for poor overall survival [hazard ratio (HR)=4.95, 95%CI=1.08-22.7; *p*=0.039; Table II]. Impact of

Table I. *The characteristics of the patients and tumours in the clinical specimen.*

	Variables	Total (n=62)
Age*		68 (38-90)
Gender	Male	35
	Female	27
BMI (kg/m^2)*		21.8 (15.3-38.9)
Tumour location	Colon	41
	Rectum	21
Tumour size (cm)*		4.0 (2.0-11.0)
Tumour depth***	pT2	11
	pT3/4	51
Nodal involvements***	pN0	31
	pN1/2/3	31
Tumour grade	tub1/tub2	58
	por/muc	4
Lymphatic invasion		38 (61.3%)
Vascular invasion		44 (71.0%)
Adjuvant chemotherapy		27 (43.5%)
BAF57 value**		0.309 (0.021-5.007)

*Median (range); **Average; ***Postoperative diagnosis of TNM classification. BMI: Body mass index.

the BAF57 value on recurrence-free survival based on univariate analysis using the Cox proportional hazards model is shown in Table III. Multivariate analysis using the Cox proportional hazards model adjusted for pathological N factor revealed that BAF57 expression was an independent risk factor for poor recurrence-free survival (HR=2.72, 95%CI=1.94-7.88; *p*=0.035; Table III).

Discussion

In the current study, CRC cell lines were observed to express BAF57 at various levels, similar to that seen in gynaecological cancers. We anticipated that BAF57 may be a risk factor for CRC malignancy and an indicator of worsening prognosis. Consistent with this, relatively high expression of BAF57 was detected in the CRC cell line WiDr, which exhibited higher invasive ability compared to that of HT-29 cells, which demonstrated relatively low expression of BAF57. In addition, suppression of BAF57 expression in WiDr cells by siRNA treatment decreased their invasive ability in a dose-dependent manner. Furthermore, analysis of surgical specimens from patients with CRC revealed that BAF57 expression was an independent prognostic factor in CRC. To the best of our knowledge, this is the first report to describe the impact of BAF57 on CRC. Our findings suggest that BAF57 may be a potential target for novel CRC therapies, as well as a biomarker for prognosis.

BAF57 is a subunit of all mammalian SWI/SNF complexes, which promotes gene expression by remodelling nucleosomes.
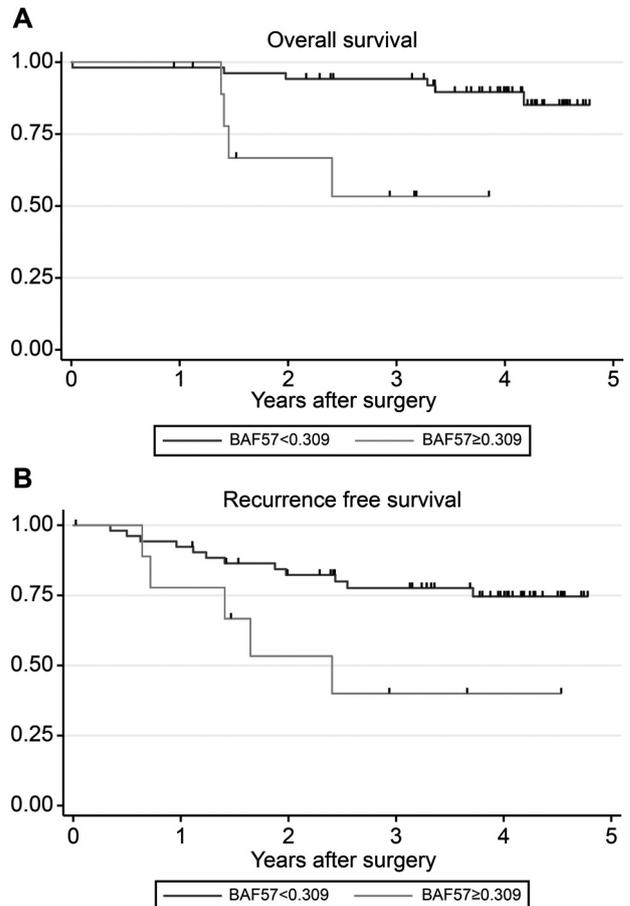
Figure 4. *Kaplan–Meier curves of long-term survival of patients with CRC relative to BAF57 expression. The patients (n=62) underwent curative resection and were divided into two groups based on BAF57 mRNA levels measured by real-time RT-PCR. A cut-off value of BAF57 mRNA relative to GAPDH between the two patient groups was set at 0.309. (A) Kaplan–Meier curve reveals 91.2% of patients with high BAF57-expressing specimens compared to 52.5% of patients with low BAF57-expressing specimens demonstrate 3-year overall survival (HR=6.52, 95%CI=1.34-25.6; p<0.001). (B) Kaplan–Meier curve reveals 76.2% of patients with high BAF57-expressing specimens compared to 39.3% of patients with low BAF57-expressing specimens demonstrate 3-year recurrence-free survival (HR=3.54, 95%CI=1.17-7.56; p=0.022).*

Numerous studies have reported various important roles of SWI/SNF complexes in epigenetic regulation during tumorigenesis, differentiation, and development (9, 19, 20). In prostate cancer, BAF57 expression has been reported to accelerate cell differentiation and migration, resulting in the induction of metastasis (13). The results of our current study suggest that high BAF57-expressing CRC exhibits accelerated cell differentiation compared with that of low BAF57-expressing CRC. It is also possible that suppression of BAF57 may result in down-regulation of the cell cycle and reduced cell invasion ability. Accordingly, BAF57 may be associated

Table II. *Univariate and multivariate analysis of overall survival using the Cox proportional hazard model.*

Variable	Univariate analysis			Multivariate analysis		
	Hazard ratio	95%CI	*p*-Value	Hazard ratio	95%CI	*p*-Value
Age*						
<68	1					
>68	2.31	0.60-8.95	0.225			
Gender						
Female	1			1		
Male	1.24	0.35-4.40	0.74	1.1	0.30-4.01	0.885
BMI (kg/m^2)*						
<21.8	1					
>21.8	1.47	0.41-5.21	0.551			
Tumour location						
Colon	1			1		
Rectum	1.31	0.37-4.65	0.675	1.25	0.33-4.79	0.746
Tumour size (cm)*						
<4.0	1			1		
>4.0	1.14	0.32-4.03	0.843	0.63	0.14-2.82	0.547
Tumour grade						
tub1, tub2	1					
por, muc	2.72	0.34-21.9	0.349			
pT stage						
pT2	1					
pT3, T4						
pN factor						
N(−)	1			1		
N(+)	1.58	0.44-5.61	0.48	2.93	0.67-12.8	0.153
Lymphatic invasion						
ly(−)	1					
ly(+)	1.64	0.42-6.34	0.476			
Vascular invasion						
v(−)	1					
v(+)	1.79	0.38-8.44	0.462			
Adjuvant chemotherapy						
Adjuvant (+)	1			1		
Adjuvant (−)	3.7	0.78-17.5	0.098	4.93	0.86-28.4	0.074
BAF57**						
<0.309	1			1		
>0.309	7.69	1.92-30.8	0.004	4.95	1.08-22.7	0.039

*Median (range); **Average. BMI: Body mass index.

with the induction of cell cycle arrest and apoptosis in CRC cells. Indeed, Hah *et al*. reported that knockdown of BAF57 contributes to the suppression of cell proliferation and promotes the accumulation of cells in the G$_2$/M phase of the cell cycle (21).

Martineti *et al*. reported that ERβ regulates cell proliferation by controlling key cell cycle modulators (22). Rudolph *et al*. revealed the loss of ERβ expression is related to CRC progression and ERβ may be a novel prognostic indicator for CRC (14). Similarly, BAF57 may also be a negative regulator of cell cycle progression. In a previous report, the induction of BAF57 promoted cell cycle arrest and apoptosis in a human breast cell line that lacked BAF57 expression (23). Collectively, BAF57 is a potential determinant of CRC malignancy through its effect on the regulation of apoptosis. However, the relationship between BAF57 and ERβ remains unknown, warranting further investigation. Moreover, herein we employed only a few select CRC cell lines, however, considering the heterogeneity of cancer cells, caution must be taken when applying the current results to clinical practice. Furthermore, there is a need to increase the number of clinical specimens that are examined.

In conclusion, our study revealed that BAF57 might be a novel candidate prognostic biomarker for CRC. Moreover, these results suggest that a novel BAF57 targeted therapy in CRC may prove promising.

Table III. *Univariate and multivariate analysis of recurrence-free survival using the Cox proportional hazard model.*

Variable	Univariate analysis			Multivariate analysis		
	Hazard ratio	95%CI	*p*-Value	Hazard ratio	95%CI	*p*-Value
Age*						
<68	1					
>68	0.76	0.29-1.98	0.579			
Gender						
Female	1					
Male	1.87	0.69-5.07	0.219			
BMI (kg/m^2)*						
<21.8	1					
>21.8	2.3	0.85-6.24	0.101			
Tumour location						
Colon	1					
Rectum	1.99	0.77-5.15	0.158			
Tumour size (cm)*						
<4.0	1					
>4.0	2.41	0.78-7.38	0.125			
Tumour grade						
tub1, tub2	1					
por, muc	0.99	0.13-7.46	0.991			
pT stage						
pT2	1					
pT3, T4						
pN factor						
N(−)	1					
N(+)	5.41	1.55-18.8	0.008	5.03	1.44-17.6	0.011
Lymphatic invasion						
ly(−)	1					
ly(+)	2.42	0.79-7.44	0.122			
Vascular invasion						
v(−)	1					
v(+)	2.66	1.01-9.43	0.148			
Adjuvant chemotherapy						
Adjuvant (+)	1					
Adjuvant (−)	0.69	0.27-1.80	0.454			
BAF57**						
<0.309	1					
>0.309	3.21	1.12-9.23	0.03	2.72	1.94-7.88	0.035

*Median (range); **Average. BMI: Body mass index.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

Hirofumi Suzumura and Masashi Tsuruta contributed equally to all aspects of this study, including designed the study, designed the experiments, analysed the data, and wrote the manuscript. Koji Okabayashi, Kohei Shigeta, Ryo Seishima, Kaoru Koishikawa, Shingo Akimoto, and Takashi Ishida performed the experiments. Takehiro Shimada and Yusuke Asada provided guidance on the experimental procedures. Hirotoshi Hasegawa and Yuko Kitagawa contributed to the conception of the study, design, and coordination, and helped draft the article. All Authors reviewed the draft manuscript and approved the final version.

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