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SMAD4 alteration associates with invasive-front pathological markers and poor prognosis in colorectal cancer

Hidehito Oyanagi,[1] Yoshifumi Shimada,[1] Masayuki Nagahashi,[1] Hiroshi Ichikawa,[1] Yosuke Tajima,[1] Kaoru Abe,[1] Masato Nakano,[1] Hitoshi Kameyama,[1] Yasumasa Takii,[2] Takashi Kawasaki,[3] Kei-Ichi Homma,[3] Yiwei Ling,[4] Shujiro Okuda,[4] Kazuaki Takabe[5,6,7,8] & Toshifumi Wakai[1]

[1]*Division of Digestive and General Surgery, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan,* [2]*Department of Surgery, Niigata Cancer Center Hospital, Niigata, Japan,* [3]*Department of Pathology, Niigata Cancer Center Hospital, Niigata, Japan,* [4]*Division of Bioinformatics, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan,* [5]*Division of Breast Surgery, Roswell Park Comprehensive Cancer Center, Buffalo, NY, USA,* [6]*Department of Surgery, University at Buffalo Jacobs School of Medicine and Biomedical Sciences, The State University of New York, Buffalo, NY, USA,* [7]*Department of Breast Surgery and Oncology, Tokyo Medical University, Tokyo, Japan, and* [8]*Department of Surgery, Yokohama City University, Yokohama, Japan*

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SMAD4 alteration associates with invasive-front pathological markers and poor prognosis in colorectal cancer

Aims: SMAD4 acts as a tumour suppressor, and the loss of SMAD4 is associated with poor prognosis in colorectal cancer (CRC) patients. Although next-generation sequencing (NGS) enabled us to detect numerous genetic alterations in a single assay, the clinical significance of *SMAD4* alteration detected with NGS has not been fully investigated. The aim of this study was to evaluate the clinicopathological characteristics and clinical significance of *SMAD4* alteration detected with NGS in CRC.

Methods and results: We retrospectively investigated 201 patients with stage I–IV CRC, by using a 415-gene panel. To analyse the relationship between *SMAD4* alteration and other clinicopathological characteristics, we evaluated clinicopathological variables, including invasive-front pathological markers: tumour budding, poorly differentiated cluster, and Crohn-like lymphoid reaction. Fifty-six patients (28%) had *SMAD4* alteration: 24 and 32 patients had *SMAD4* mutation and deletion, respectively. *SMAD4* alteration was significantly associated with T category ($P = 0.027$), N category ($P = 0.037$), M category ($P = 0.028$), and invasive-front pathological markers, such as poorly differentiated cluster grade 3 ($P = 0.020$) and absence of Crohn-like lymphoid reaction ($P = 0.004$). Immunohistochemistry revealed that *SMAD4* alteration was significantly associated with loss of SMAD4 ($P = 0.023$). In 90 patients with stage I–III disease, *SMAD4* alteration was significantly associated with poor prognosis for relapse-free and overall survival ($P = 0.047$; $P = 0.022$, respectively). Conversely, in 111 patients with stage IV disease, *SMAD4* alteration was not significantly associated with overall survival.

Conclusion: *SMAD4* alteration is associated with invasive-front pathological markers and poor prognosis in stage I–III CRC patients.

Keywords: colorectal cancer, Crohn-like lymphoid reaction, genetic alteration, immunohistochemistry, next-generation sequencing, poorly differentiated cluster, SMAD4, TGF-β

Address for correspondence: Yoshifumi Shimada, Division of Digestive and General Surgery, Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachi-dori, Chuo-ku, Niigata City, Niigata 951-8510, Japan. e-mail: shimaday@med.niigata-u.ac.jp

Introduction

Colorectal cancer (CRC) is one of the most frequent cancers and is a common cause of cancer-related deaths.[1] Over the last decade, the clinical outcome for patients with CRC has improved. For metastatic CRC, the median overall survival (OS) of patients treated both in phase III trials and in large observational series or registries is close to 30 months, and more than double that of 20 years ago.[2] Although it is unclear which improvements and strategic changes in the treatment and management of CRC patients have contributed to the treatment outcome, several emerging genomic biomarkers are considered to be one of the contributors to improved clinical outcomes. However, there were 1.4 million new CRC cases with 69,400 deaths worldwide in 2012.[1] Hence, novel approaches for the diagnosis and treatment of CRC are needed.

Next-generation sequencing (NGS) has the ability to identify clinically important genomic biomarkers in several signalling pathways,[3,4] and is considered to be a useful tool for genetic-based diagnosis.[5] In CRC, several molecular biomarkers have been clinically used as both predictive markers of anti-epidermal growth factor receptor therapy[6,7] and prognostic markers of survival outcome.[2,8–10] This is especially so regarding genetic alterations in the mitogen-activated protein kinase signalling pathway, such as *KRAS*, *NRAS*, and *BRAF* mutations, which are widely recognised to be clinically important CRC biomarkers.[2,9,10]

The transforming growth factor (TGF)-β signalling pathway plays a critical role in tumour progression.[11,12] SMAD proteins are key signal transducers of the TGF-β pathway, and are essential for TGF-β function,[13] which includes suppression of cell growth, proliferation, differentiation, motility, apoptosis and epithelial–mesenchymal transition (EMT).[14,15] The TGF-β family includes 30 proteins that signal via a common mechanism through serine/threonine kinase transmembrane receptors.[16] Loss of expression and mutational defects of these receptors are prominent mechanisms of escape from the biological control of TGF-β in CRC.[16]

SMAD family member 4 (SMAD4) is localised to band 18q21, is phosphorylated and activated by transmembrane serine-threonine receptor kinase, and is one of the key mediators of the TGF-β pathway.[17] The protein acts as a tumour suppressor and inhibits epithelial cell proliferation, and the loss of SMAD4 is associated with poor prognosis and resistance to 5-fluorouracil (5-FU) in CRC patients.[18–20] Sporadic *SMAD4* mutation has been reported in 2.1–20.0% of CRCs.[3,21–23] On comparison of genetic alterations of primary tumour with those of metastatic tumour, *SMAD4* mutation is one of the most frequent genetic alterations in metastatic CRC, suggesting that *SMAD4* is involved in clonal evolution in CRC.[24] Recent advances in NGS technology have enabled the detection of numerous genetic alterations, such as single-nucleotide polymorphisms (SNPs), small insertions/deletions (indels), copy number variation, and translocations; however, the clinical significance of *SMAD4* alterations detected with NGS has not been fully investigated.

Recently, it was reported that histological patterns at the invasive front were associated with tumour biology and oncological outcomes in CRC.[25] Tumour budding, which is defined as fewer than five tumour cells, and poorly differentiated clusters (PDC), which are defined as five or more cancer cells with no gland formation, are often observed at the invasive front, and are associated with lymph node metastasis,[26,27] drug resistance[28] and poor prognosis[29,30] in CRC. Crohn-like lymphoid reaction and tumour-infiltrating lymphocytes, which represent the host immune response at the invasive front, are associated with better prognosis.[31–33] However, few studies have evaluated the association between genetic alterations and histological patterns, including such invasive-front markers. In this analysis, we evaluated the association between *SMAD4* alteration and pathological features, focusing on invasive-front pathological markers in CRC.

The aim of this study was to evaluate the clinicopathological characteristics and clinical significance of *SMAD4* alteration detected with NGS in CRC.

Materials and methods

PATIENTS

This retrospective analysis was performed in accordance with the Helsinki Declaration, and the Ethics Committee of the School of Medicine, Niigata University, approved the study protocol. All methods were performed in accordance with the relevant guidelines and regulations, and written informed consent was obtained from all patients. We enrolled a total of 201 patients diagnosed with stage I–IV CRC according to the American Joint Committee on Cancer 8th edition[9] who had undergone primary tumour resection between 2009 and 2015 at Niigata University Medical and Dental Hospital or Niigata Cancer Centre Hospital. Patients with familial adenomatous

polyposis or inflammatory bowel disease were excluded. No patients had received neoadjuvant chemoradiation.

NGS OF RESECTED TUMOURS

Formalin-fixed paraffin-embedded (FFPE) samples were used for NGS, and genetic alterations, including those of *SMAD4*, were evaluated. An independent pathologist evaluated tumour content on haematoxylin and eosin (H&E)-stained sections for each study sample to ensure that >50% tumour content was present. When applicable, unstained sections were macrodissected to enrich for tumour content, and DNA was extracted with a BioStic FFPE Tissue DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA). All sample preparation, NGS and bioinformatics analysis were performed in a Clinical Laboratory Improvement Amendments/College of American Pathologists-accredited laboratory (KEW, Cambridge, MA, USA). DNA fragment (50–150 ng) libraries were prepared and enriched for the 415-gene panel with CancerPlex (KEW).[34–36] CancerPlex Version 3.0 is a clinically validated 415-gene panel enriched for coding regions and selected introns of genes with a known association with cancer. Sequencing was performed on the Illumina MiSeq and NextSeq platforms (Illumina, San Diego, CA, USA) with an average sequencing depth of ×500. Genomic data were then processed by use of a proprietary bioinformatics platform and knowledge base to identify multiple classes of genomic abnormalities, including SNPs, small indels, copy number variation, and translocations. A threshold of 10% allelic fraction was used for SNPs and indels, and thresholds of >2.5-fold (gain) and 0.5-fold (loss) were used. Tumours were tested for the presence of microsatellite instability (MSI) on the basis of an extended loci panel. In addition to the Bethesda panel, a collection of 950 regions consisting of tandem repeats of one, two or three nucleotides with a minimum length of 10 bases was used. The number of indels within the region of interest was calculated, and tumours were classified as MSI-high (MSI-H) or microsatellite-stable.

SMAD4 MUTATIONAL STATUS ANALYSIS

In the present analysis, we compared *SMAD4* mutational prevalence, spectrum and frequency between our cohort and The Cancer Genome Atlas (TCGA) samples. The mutation information for the TCGA CRC-sequenced samples ($n = 73$) within the members of the TGF-β signalling pathway, including *ACVR2A*, *TGFBR2*, *SMAD2*, and *SMAD4*, was obtained from the cBioPortal[37] to assess mutation frequency. The frequency of the mutations in the TGF-β signalling pathway was quantified with the R-language (https://www.r-project.org/).

SMAD4 ALTERATION AND OTHER CLINICOPATHOLOGICAL CHARACTERISTICS

To analyse the relationship between *SMAD4* alteration and other clinicopathological characteristics, 21 clinicopathological variables were examined in all 201 patients (Table 1). One of the authors (Y.S.) evaluated invasive-front pathological markers: tumour budding, PDC, and Crohn-like lymphoid reaction.[25] Tumour budding was defined as the presence of individual cells and small clusters (fewer than five) of tumour cells in a field containing maximum clusters. A PDC was defined as the presence of five or more cancer cells with no gland formation in a field containing maximum clusters. Tumour budding was evaluated according to the recommendations of the International Tumor Budding Consensus Conference 2016.[38] The clusters of tumour budding were counted under a microscopic field measuring 0.785 mm^2 observed through a ×20 objective lens [WHK ×10 ocular lens (Olympus, Tokyo, Japan)].[38,39] Tumours with <5, 5–9 and ≥10 clusters were classified as grade Bd1, Bd2, and Bd3, respectively. Clusters of PDC were also counted under a 0.785-mm^2 microscopic field observed through a ×20 objective lens (WHK ×10 ocular lens). Tumours with <5, 5–9 and ≥10 clusters were classified as grade G1, G2, and G3, respectively.[25,30] Crohn-like lymphoid reaction was defined as nodular lymphoid aggregates of ≥1 mm at the tumour periphery, and was classified as absent or present.[25] Histopathological markers associated with deficiency of mismatch repair genes, such as medullary type, mucinous type, and tumour-infiltrating lymphocytes, were analysed with a previously reported method.[40]

IMMUNOHISTOCHEMISTRY AND PATHOLOGICAL EVALUATION OF SMAD4 EXPRESSION

Each FFPE sample used for NGS analysis was re-cut, generating 4-μm-thick sections, and three serial sections were assigned for H&E staining, anti-SMAD4 staining, and as a negative control. Immunohistochemistry was performed according to a previous report, with anti-SMAD4 mouse monoclonal antibody (clone B-8; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 200 mg/ml, diluted 1:1000).[18] With this antibody, SMAD4 staining is both nuclear and cytoplasmic. We used a semiquantitative assessment of

Table 1. *SMAD4* status and other clinicopathological characteristics

Variable	*SMAD4* status: Wild *n* (%)	*SMAD4* status: Altered *n* (%)	*P*-value
Age (years)			
<65	68 (34)	32 (16)	0.211
≥65	77 (38)	24 (12)	
Sex			
Male	80 (40)	37 (18)	0.202
Female	65 (32)	19 (10)	
Location			
Right side	37 (18)	19 (10)	0.292
Left side	108 (54)	37 (18)	
Tumour size (mm)			
<50	73 (36)	15 (8)	**0.003**
≥50	72 (36)	41 (20)	
T category			
T1, T2	22 (11)	2 (1)	**0.027**
T3, T4	123 (61)	54 (27)	
Histopathological grading			
G1, G2	105 (52)	42 (21)	0.859
G3	40 (20)	14 (7)	
Lymphatic invasion			
Absence	61 (30)	18 (9)	0.259
Presence	84 (42)	38 (19)	
Venous invasion			
Absence	40 (20)	8 (4)	0.064
Presence	105 (52)	48 (24)	
N category			
N0	49 (24)	10 (5)	**0.037**
N1, N2	96 (48)	46 (23)	
M category			
M0	72 (36)	18 (9)	**0.028**
M1	73 (36)	38 (19)	
Tumour budding			
Bd1, Bd2	118 (59)	40 (20)	0.129
Bd3	27 (13)	16 (8)	
Tumour budding			
Bd1	67 (33)	22 (11)	0.430
Bd2, Bd3	78 (39)	34 (17)	
Poorly differentiated cluster			
G1, G2	102 (51)	29 (14)	**0.020**
G3	43 (21)	27 (14)	
Microsatellite instability			
High	14 (7)	1 (1)	0.072
Stable	131 (65)	55 (27)	
Medullary type			
Presence	3 (2)	2 (1)	0.620
Absence	142 (70)	54 (27)	
Mucinous type			
Presence	9 (4)	6 (3)	0.368
Absence	136 (68)	50 (25)	
Crohn-like lymphoid reaction			
Presence	29 (14)	2 (1)	**0.004**
Absence	116 (58)	54 (27)	
Tumour-infiltrating lymphocytes*			
Presence	27 (14)	9 (4)	0.838
Absence	118 (59)	47 (23)	
KRAS codon 12/13			
Wild-type	105 (52)	37 (18)	0.392
Mutant	40 (20)	19 (10)	
NRAS codon 12/13			
Wild-type	144 (71)	56 (28)	0.999
Mutant	1 (1)	0 (0)	

Table 1. *(Continued)*

Variable	*SMAD4* status: Wild *n* (%)	*SMAD4* status: Altered *n* (%)	*P*-value
BRAF V600E			
Wild-type	136 (68)	52 (26)	0.758
Mutant	9 (4)	4 (2)	
SMAD2			
Wild-type	142 (71)	37 (18)	**<0.001**
Mutant/deleted	3 (2)	19 (9)	

Fisher's exact test.
Bold type means *P*-value <0.05.
*Cut-off value: 10 lymphocytes per five high-power fields.

SMAD4 staining intensity as follows: intense (strong) expression as in non-tumour mucosa; decreased (weak) expression, less intense than in normal mucosa; and absence of staining (loss).[18] Among the absence of staining (loss), clonal loss was defined as loss of SMAD4 expression in a clonal pattern and seeming to involve whole glands or groups of glands, but never single dispersed tumour cells.[18] In each tumour, the percentage of tumour cells showing loss of expression was separately scored. According to a previous report, cases with ≥5% neoplastic cells with no SMAD4 expression were classified as having loss of SMAD4 expression. Cases with >95% of neoplastic cells with strong expression were classified as having strong SMAD4 expression, and all others were classified as having weak SMAD4 expression.[18]

STATISTICAL ANALYSIS

Statistical analyses were performed with IBM SPSS STATISTICS 22 (IBM Japan, Tokyo, Japan). *SMAD4* alteration and each clinicopathological variable were evaluated with Fisher's exact test. Relapse-free survival (RFS) and OS rates were estimated with Kaplan–Meier analysis. A log-rank test was used to assess for a significant difference between *SMAD4* wild-type and *SMAD4* alteration. *P*-values of <0.05 were considered to be statistically significant.

Results

ALTERATION OF *SMAD4* IN JAPANESE CRC

To date, there have been no reports regarding genetic alterations in the TGF-β pathway among Japanese CRC patients; hence, we evaluated the genetic alterations of *TGFBR2*, *ACVR2A*, *SMAD2*, and *SMAD4*, and compared them with TCGA data. Fifty-six patients (28%) had *SMAD4* alteration: 24 and 32 patients had *SMAD4* mutation and deletion, respectively. *SMAD4* deletion was observed more in the Japanese cohort than in TCGA samples (Figures 1 and 2).

SMAD4 ALTERATION AND CLINICOPATHOLOGICAL CHARACTERISTICS

We investigated the association between *SMAD4* alteration and tumour progression, and evaluated the association between *SMAD4* alteration and pathological features, including invasive-front markers such as tumour budding, PDC, Crohn-like lymphoid reaction, and tumour-infiltrating lymphocytes. *SMAD4* alteration was significantly associated with tumour size ($P = 0.003$), T category ($P = 0.027$), N category ($P = 0.037$), M category ($P = 0.028$), PDC G3 ($P = 0.020$; Figure 3), absence of Crohn-like lymphoid reaction ($P = 0.004$), and *SMAD2* alteration ($P < 0.001$; Table 1). Regarding MSI status, 15 of 201 (7%) patients were MSI-high. Of the 15 MSI-high patients, only one patient had *SMAD4* alteration ($P = 0.072$; Table 1), suggesting that MSI-high and *SMAD4* alteration might represent distinct subgroups in CRC.

Regarding SMAD4 expression evaluated with immunohistochemistry, 45 patients showed loss of SMAD4 expression, whereas 50 and 106 patients showed strong and weak expression, respectively (Figure 4). Clonal loss was observed in nine of 45 (20%) patients with loss of SMAD4 expression. *SMAD4* alteration was significantly associated with loss of SMAD4 expression ($P = 0.023$; Table 2). Nineteen of 56 (34%) patients with *SMAD4* alteration showed loss of SMAD4 expression (Table 3), whereas 26 of 145 (18%) patients with wild-type *SMAD4* showed loss of SMAD4 expression.

PROGNOSTIC SIGNIFICANCE OF *SMAD4* ALTERATION IN STAGE I–III AND STAGE IV PATIENTS

In 90 patients with stage I–III disease, *SMAD4* alteration was significantly associated with poor prognosis for RFS (Figure 5) and OS (Figure 6A) ($P = 0.047$ and $P = 0.022$, respectively). Conversely, in 111 patients with stage IV disease, *SMAD4* alteration was not significantly associated with OS (Figure 6B).

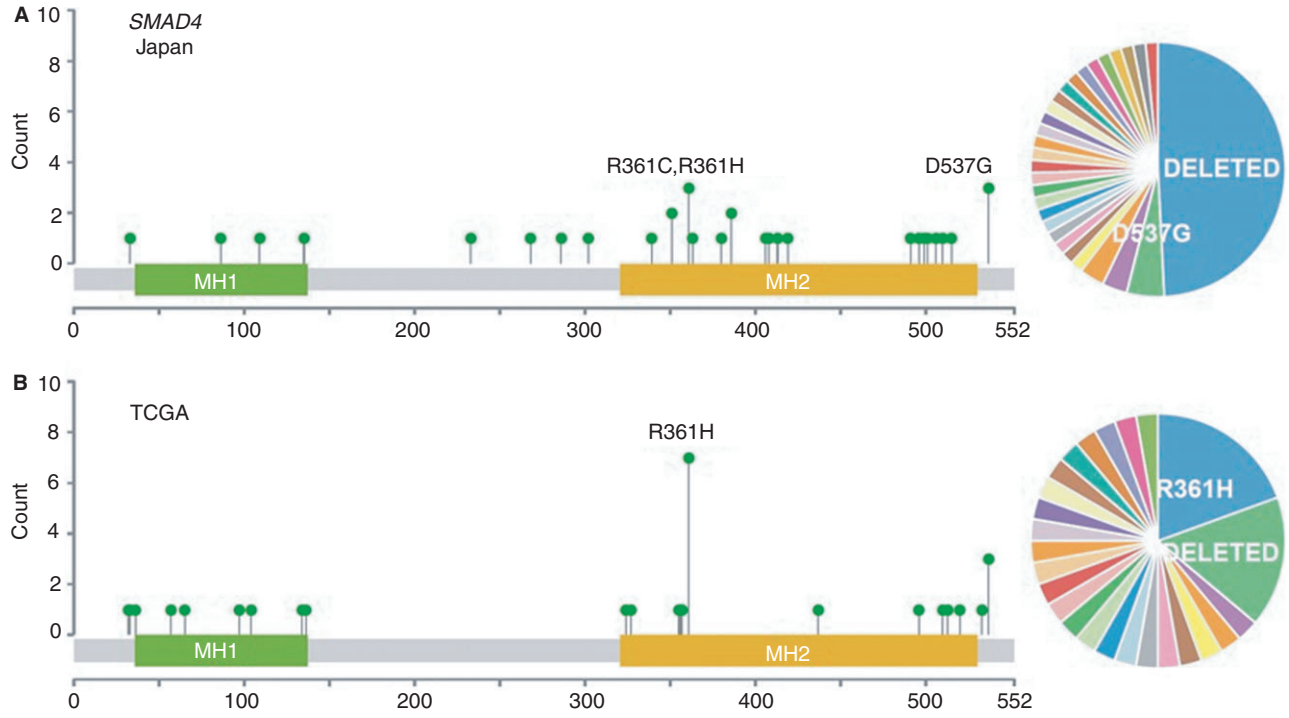

Figure 1. Comparison of the prevalence and spectrum of *SMAD4* mutations between the Japanese cohort and The Cancer Genome Atlas (TCGA) data. **A**, Japanese samples. **B**, TCGA samples.

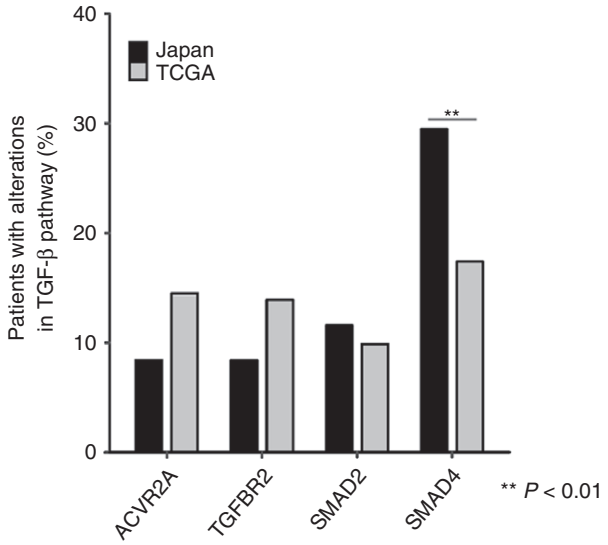

Figure 2. Comparison of the frequency of genetic alterations in the transforming growth factor-β pathway between the Japanese cohort and The Cancer Genome Atlas data.

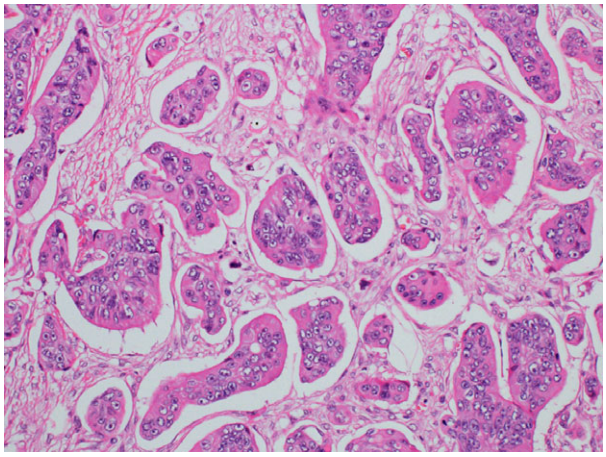

Figure 3. Poorly differentiated clusters. Haematoxylin and eosin staining. [Colour figure can be viewed at wileyonlinelibrary.com]

Discussion

In the present analysis, there were three main findings regarding *SMAD4* alteration in CRC. First, *SMAD4* alteration was significantly associated with high TNM categories and invasive-front pathological markers associated with prognosis, such as PDC and Crohn-like lymphoid reaction. Second, *SMAD4* alteration was significantly associated with loss of SMAD4 expression. Third, *SMAD4* alteration was a poor prognostic factor in patients with stage I–III disease. These results imply that *SMAD4* inactivation induced by

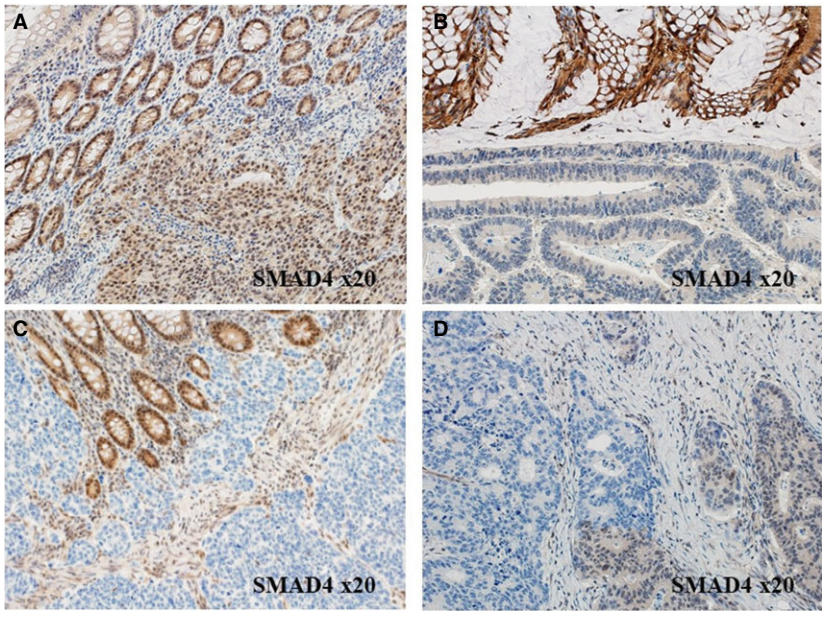

Figure 4. Patterns of SMAD4 expression in colorectal cancer: anti-SMAD4 staining. **A**, Strong expression. **B**, Weak expression. **C**, Complete loss (note positive stromal cells and normal epithelial cells). **D**, Clonal loss. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 2. Comparison between *SMAD4* gene and SMAD4 immunohistochemistry (IHC) statuses

	SMAD4 gene status	
	Wild-type *n* (%)	Altered *n* (%)
SMAD4 IHC status		
Strong/weak	119 (60)	37 (18)
Loss	26 (13)	19 (9)

Fisher's exact test.
$N = 201$, $P = 0.023$.

the genetic alteration has a key role in tumour progression of CRC, and *SMAD4* alteration, detected with NGS, is a possible prognostic factor in stage I–III CRC.

In this analysis, we investigated *SMAD4* mutation and deletion by using NGS, and found that 56 patients (28%) had *SMAD4* alteration: 24 and 32 patients had *SMAD4* mutation and deletion, respectively. SMAD4 has an architecture consisting of two globular domains connected by a linker of variable length and sequence. The N-terminal MAD homology 1 (MH1) domain is a DNA-binding domain, and the C-terminal MAD homology 2 (MH2) domain is a protein–protein interaction module mediating SMAD multimerisation and transactivation. Mutations in the MH1 domain have been shown to alter SMAD4 stability, DNA binding, and nuclear translocation; and mutations in the MH2 domain mainly affect residues close to the protein interface involved in homo-oligomerization and hetero-oligomerization of SMAD4 with R-SMAD proteins, which are required for activation.[21] Previous analyses showed that the majority of *SMAD4* alterations in CRC were missense mutations in the MH2 domain,[21–24] which is consistent with our results.

The loss of SMAD4 expression has been shown to be associated with metastasis, resistance to 5-FU, and poor prognosis.[18–20] Yan *et al.* reported that reduced SMAD4 expression was strongly associated with poor OS and RFS in stage III patients, and the incidence of SMAD4 loss increases according to TNM stage, providing strong support for the notion that SMAD4 loss is involved in tumour progression.[18] Zhang *et al.* reported that loss of SMAD4 expression induces resistance to 5-FU-based therapies through Akt pathway activation.[20] However, the role of *SMAD4* alteration affecting SMAD4 protein expression remains to be elucidated.[21] In the current analysis, we found that *SMAD4* alteration was significantly associated with loss of SMAD4 expression in CRC.

In previous reports, SMAD4 expression was evaluated by the use of surgically resected specimens, and not biopsy specimens.[18–20] We also evaluated SMAD4 expression by using surgically resected specimens, and found that clonal loss was present in nine of 45 (20%) patients with loss of SMAD4 expression. We

Table 3. Nineteen patients with both *SMAD4* alteration and loss of SMAD4 expression

Case no.	*SMAD4* gene status	SMAD4 IHC status	Percentage of SMAD4 loss area
1	Deleted	Loss	30
2	Deleted	Loss	100
3	Deleted	Loss	80
4	Deleted	Loss	100
5	Deleted, R515I	Loss	30
6	G510R	Loss	10
7	Deleted	Loss	80
8	Deleted, E33X	Loss	100
9	Deleted	Loss	90
10	G491fs	Loss	95
11	Deleted	Loss	100
12	413_416del, F408fs	Loss	100
13	Deleted	Loss	100
14	Deleted	Loss	20
15	W302X	Loss	100
16	Deleted	Loss	20
17	Deleted	Loss	20
18	L109fs	Loss	100
19	G86V	Loss	100

IHC, immunohistochemistry.

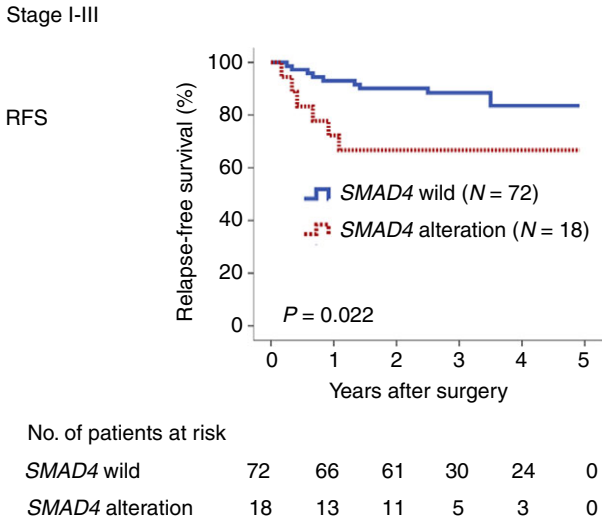

Figure 5. Kaplan–Meier curves of overall survival and relapse-free survival for stage I–III patients. [Colour figure can be viewed at wileyonlinelibrary.com]

recommend that SMAD4 expression should be evaluated by the use of surgically resected rather than biopsy specimens, considering the existence of clonal loss of SMAD4 expression.

TGF-β signalling has been shown to regulate normal colonic epithelial differentiation and to inhibit normal intestinal epithelial cell proliferation.[11,12] It is widely believed that, in human CRC, loss of differentiation is induced under the EMT programme at the tumour–host interface, enabling cellular detachment, dissemination, and eventual metastasis.[14,15] EMT signalling is considered to introduce tumour cells to PDCs in the microenvironment of the tumour front.[41] In the present analysis, we found that PDCs were significantly more frequent in patients with *SMAD4* alteration, implying that *SMAD4* alteration might be one of the triggers of EMT in CRC.

The lymphocytic reaction observed at the invasive front in CRC has been recognised as an indicator of host immune responses to tumour cells.[32] Several studies have shown that Crohn-like lymphoid reaction is a feature associated with favourable outcomes and MSI-high CRC.[31–33] In this analysis, we evaluated the association between *SMAD4* alteration and the lymphatic reaction observed at the invasive front, i.e. tumour-infiltrating lymphocytes and Crohn-like lymphoid reaction, and showed that *SMAD4* alteration was significantly associated with the absence of Crohn-like lymphoid reaction. We speculate that the absence of Crohn-like reaction is a characteristic pathological feature in patients with *SMAD4* alteration that affects the prognosis of patients with *SMAD4* alteration.

Few studies have evaluated the association between histological patterns and *SMAD4* alteration in CRC. Previous studies showed that *SMAD4* mutation was frequently observed in mucinous carcinoma,[23,42] and was associated with the aggressive phenotype of high-grade mucinous carcinoma in CRC.[43] In the current study, we focused on the pathological features of both tumour aggressiveness and the host immune response at the invasive front of tumours. We demonstrated that *SMAD4* alteration was significantly associated with PDC grade 3, which has been reported as an aggressive phenotype in CRC,[25,27,28,30,41] and the absence of Crohn-like lymphoid reaction, which has been recognised as an indicator of host immune responses to tumour cells in

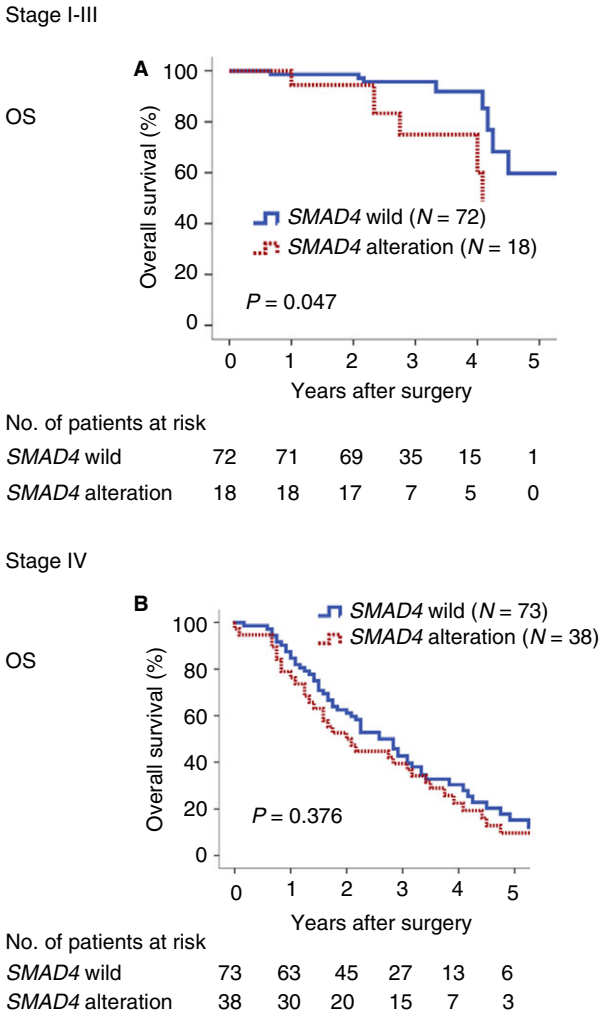

Figure 6. Kaplan–Meier curves of overall survival. **A**, Stage I–III patients. **B**, Stage IV patients. [Colour figure can be viewed at wileyonlinelibrary.com]

CRC.[31] These results suggest that *SMAD4* mutation might be associated with both tumour aggressiveness and suppression of the host immune response, resulting in poor prognosis in CRC patients.

This analysis has several limitations. First, this was a retrospective analysis performed at two institutions, and included a small number of patients. Second, the cohort was not consecutive. Hence, we cannot draw a definitive conclusion regarding the prognostic impact of *SMAD4* alteration in CRC. However, to the best of our knowledge, this is the first report to demonstrate the clinical significance of *SMAD4* alteration by evaluating the pathological features and focusing on invasive-front pathological markers in CRC.

In conclusion, we found that *SMAD4* alteration was associated with invasive-front pathological markers and poor prognosis in stage I–III CRC, suggesting that *SMAD4* alteration represents a distinct molecular subtype in CRC. Further studies are needed to evaluate these findings and the clinical significance of *SMAD4* alteration in CRC.

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Conflicts of interest

The authors state that they have no conflicts of interest.

Author contributions

Conception and design: Y. Shimada. Development of methodology: Y. Shimada and T. Wakai. Acquisition of data (acquired and managed patients, provided facilities, etc.): Y. Shimada, M. Nagahashi, H. Ichikawa, Y. Tajima, K. Abe, M. Nakano, H. Kameyama, Y. Takii, T. Kawasaki, and K. Homma. Analysis and interpretation of data (e.g. statistical analysis, biostatistics, and computational analysis): Y. Ling and S. Okuda. Writing, review and/or revision of the manuscript: H. Oyanagi, Y. Shimada, K. Takabe, and T. Wakai. Administrative, technical or material support (i.e. reporting or organising data, and constructing databases): Y. Ling and S. Okuda. Study supervision: K. Takabe and T. Wakai.

References

1. World Health Organization. *GLOBOCAN 2012: Estimated Cancer Incidence, Mortality and Prevalence Worldwide in 2012*. Available from: http://globocan.iarc.fr/Pages/fact_sheets_population.aspx (accessed 14 July 2018).
2. Van Cutsem E, Cervantes A, Adam R, *et al.* ESMO consensus guidelines for the management of patients with metastatic colorectal cancer. *Ann. Oncol.* 2016; **27**; 1386–1422.
3. Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer. *Nature* 2012; **487**; 330–337.
4. Cancer Genome Atlas Research Network. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature* 2008; **455**; 1061–1068.
5. Davey JW, Hohenlohe PA, Etter PD, *et al.* Genome-wide genetic marker discovery and genotyping using next-generation sequencing. *Nat. Rev. Genet.* 2011; **12**; 499–510.
6. Douillard JY, Oliner KS, Siena S, *et al.* Panitumumab-FOLFOX4 treatment and RAS mutations in colorectal cancer. *N. Engl. J. Med.* 2013; **369**; 1023–1034.

7. Van Cutsem E, Lenz HJ, Köhne CH, *et al.* Fluorouracil, leucovorin, and irinotecan plus cetuximab treatment and RAS mutations in colorectal cancer. *J. Clin. Oncol.* 2015; **33**; 692–700.
8. Allegra CJ, Jessup JM, Somerfield MR, *et al.* American Society of Clinical Oncology provisional clinical opinion: testing for KRAS gene mutations in patients with metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy. *J. Clin. Oncol.* 2009; **27**; 2091–2096.
9. Amin MB, Edge S, Greene F *et al.* eds. *AJCC cancer staging manual*. 8th ed. New York, NY: Springer International, 2017.
10. National Comprehensive Cancer Network. *NCCN clinical practice guidelines in oncology-rectal cancer* (version 1. 2018). Available from: https://www.nccn.org/professionals/physician_gls/pdf/colon.pdf (accessed 2 March 2018).
11. Massagué J. TGFbeta in cancer. *Cell* 2008; **134**; 215–230.
12. Ikushima H, Miyazono K. TGFbeta signalling: a complex web in cancer progression. *Nat. Rev. Cancer* 2010; **10**; 415–424.
13. Wang H, Rajan S, Liu G, Chakrabarty S. Transforming growth factor beta suppresses beta-catenin/Wnt signaling and stimulates an adhesion response in human colon carcinoma cells in a Smad4/DPC4 independent manner. *Cancer Lett.* 2008; **264**; 281–287.
14. Pickup M, Novitskiy S, Moses HL. The roles of TGFβ in the tumour microenvironment. *Nat. Rev. Cancer* 2013; **13**; 788–799.
15. Drabsch Y, ten Dijke P. TGF-β signalling and its role in cancer progression and metastasis. *Cancer Metastasis Rev.* 2012; **31**; 553–568.
16. Jung B, Staudacher JJ, Beauchamp D. Transforming growth factor β superfamily signaling in development of colorectal cancer. *Gastroenterology* 2017; **152**; 36–52.
17. Hahn SA, Schutte M, Hoque AT, *et al.* DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. *Science* 1996; **271**; 350–353.
18. Yan P, Klingbiel D, Saridaki Z, *et al.* Reduced expression of SMAD4 is associated with poor survival in colon cancer. *Clin. Cancer Res.* 2016; **22**; 3037–3047.
19. Kozak MM, von Eyben R, Pai J, *et al.* Smad4 inactivation predicts for worse prognosis and response to fluorouracil-based treatment in colorectal cancer. *J. Clin. Pathol.* 2015; **68**; 341–345.
20. Zhang B, Zhang B, Chen X, *et al.* Loss of Smad4 in colorectal cancer induces resistance to 5-fluorouracil through activating Akt pathway. *Br. J. Cancer* 2014; **110**; 946–957.
21. Mehrvarz Sarshekeh A, Advani S, Overman MJ, *et al.* Association of SMAD4 mutation with patient demographics, tumor characteristics, and clinical outcomes in colorectal cancer. *PLoS ONE* 2017; **12**; e0173345.
22. Malapelle U, Pisapia P, Sgariglia R, *et al.* Less frequently mutated genes in colorectal cancer: evidences from next-generation sequencing of 653 routine cases. *J. Clin. Pathol.* 2016; **69**; 767–771.
23. Fleming NI, Jorissen RN, Mouradov D, *et al.* SMAD2, SMAD3 and SMAD4 mutations in colorectal cancer. *Cancer Res.* 2013; **73**; 725–735.
24. Goswami RS, Patel KP, Singh RR, *et al.* Hotspot mutation panel testing reveals clonal evolution in a study of 265 paired primary and metastatic tumors. *Clin. Cancer Res.* 2015; **21**; 2644–2651.
25. Konishi T, Shimada Y, Lee LH, *et al.* Poorly differentiated clusters predict colon cancer recurrence: an in-depth comparative analysis of invasive-front prognostic markers. *Am. J. Surg. Pathol.* 2018; **42**; 705–714.
26. Lee SJ, Kim A, Kim YK, *et al.* The significance of tumor budding in T1 colorectal carcinoma: the most reliable predictor of lymph node metastasis especially in endoscopically resected T1 colorectal carcinoma. *Hum. Pathol.* 2018; **78**; 8–17.
27. Ueno H, Hase K, Hashiguchi Y, *et al.* Novel risk factors for lymph node metastasis in early invasive colorectal cancer: a multi-institution pathology review. *J. Gastroenterol.* 2014; **49**; 1314–1323.
28. Tajima Y, Shimada Y, Kameyama H, *et al.* Association between poorly differentiated clusters and efficacy of 5-fluorouracil-based adjuvant chemotherapy in stage III colorectal cancer. *Jpn. J. Clin. Oncol.* 2017; **47**; 313–320.
29. Ryan É, Khaw YL, Creavin B, *et al.* Tumor budding and PDC grade are stage independent predictors of clinical outcome in mismatch repair deficient colorectal cancer. *Am. J. Surg. Pathol.* 2018; **42**; 60–68.
30. Ueno H, Hase K, Hashiguchi Y, *et al.* Site-specific tumor grading system in colorectal cancer: multicenter pathologic review of the value of quantifying poorly differentiated clusters. *Am. J. Surg. Pathol.* 2014; **38**; 197–204.
31. Ueno H, Hashiguchi Y, Shimazaki H, *et al.* Objective criteria for Crohn-like lymphoid reaction in colorectal cancer. *Am. J. Clin. Pathol.* 2013; **139**; 434–441.
32. Rozek LS, Schmit SL, Greenson JK, *et al.* Tumor-infiltrating lymphocytes, Crohn's-like lymphoid reaction, and survival from colorectal cancer. *J. Natl Cancer Inst.* 2016; **108**; djw027.
33. Kim JH, Kim KJ, Bae JM, *et al.* Comparative validation of assessment criteria for Crohn-like lymphoid reaction in colorectal carcinoma. *J. Clin. Pathol.* 2015; **68**; 22–28.
34. Nagahashi M, Wakai T, Shimada Y, *et al.* Genomic landscape of colorectal cancer in Japan: clinical implications of comprehensive genomic sequencing for precision medicine. *Genome Med.* 2016; **8**; 136.
35. Shimada Y, Yagi R, Kameyama H, *et al.* Utility of comprehensive genomic sequencing for detecting HER2-positive colorectal cancer. *Hum. Pathol.* 2017; **66**; 1–9.
36. Shimada Y, Kameyama H, Nagahashi M, *et al.* Comprehensive genomic sequencing detects important genetic differences between right-sided and left-sided colorectal cancer. *Oncotarget* 2017; **8**; 93567–93579.
37. Cerami E, Gao J, Dogrusoz U, *et al.* The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov.* 2012; **2**; 401–404.
38. Lugli A, Kirsch R, Ajioka Y, *et al.* Recommendations for reporting tumor budding in colorectal cancer based on the International Tumor Budding Consensus Conference (ITBCC) 2016. *Mod. Pathol.* 2017; **30**; 1299–1311.
39. Watanabe T, Muro K, Ajioka Y, *et al.* Japanese Society for Cancer of the Colon and Rectum (JSCCR) guidelines 2016 for the treatment of colorectal cancer. *Int. J. Clin. Oncol.* 2018; **23**; 1–34.
40. Bosman FT, Carneiro F, Hruban RH, Theise ND eds. *World Health Organization classification of tumours of the digestive system*. 4th ed. Lyon: IARC Press, 2010.
41. Ueno H, Shinto E, Kajiwara Y, *et al.* Prognostic impact of histological categorisation of epithelial–mesenchymal transition in colorectal cancer. *Br. J. Cancer* 2014; **111**; 2082–2090.
42. Shia J, Schultz N, Kuk D, *et al.* Morphological characterization of colorectal cancers in The Cancer Genome Atlas reveals distinct morphology–molecular associations: clinical and biological implications. *Mod. Pathol.* 2017; **30**; 599–609.
43. Yoshioka Y, Togashi Y, Chikugo T, *et al.* Clinicopathological and genetic differences between low-grade and high-grade colorectal mucinous adenocarcinomas. *Cancer* 2015; **121**; 4359–4368.