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

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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

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Introduction

Colorectal cancer (CRC) is one of the most frequent cancers and is a common cause of cancer-related deaths. 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. 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. 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, and is considered to be a useful tool for genetic-based diagnosis. In CRC, several molecular biomarkers have been clinically used as both predictive markers of anti-epidermal growth factor receptor therapy and prognostic markers of survival outcome. 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.

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

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. 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. Sporadic SMAD4 mutation has been reported in 2.1–20.0% of CRCs. 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. 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. 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, drug resistance and poor prognosis 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. 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 clinico-pathological 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 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). 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 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 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. 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. The clusters of tumour budding were counted under a microscopic field measuring 0.785 mm² observed through a ×20 objective lens (WHK ×10 ocular lens (Olympus, Tokyo, Japan)). 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-mm² 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. Crohn-like lymphoid reaction was defined as nodular lymphoid aggregates of ≥1 mm at the tumour periphery, and was classified as absent or present. 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.

**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). 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 | P-value |
|-------------------------------|--------------|---------|
|                              | Wild n (%)   | Altered n (%) |
| 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)  |

Table 1. (Continued)

| Variable                      | SMAD4 status | P-value |
|------------------------------|--------------|---------|
|                              | Wild n (%)   | Altered n (%) |
| 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)    |

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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). 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. 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.

**Table 1. (Continued)**

| Variable     | SMAD4 status |         |         | P-value |
|--------------|--------------|---------|---------|---------|
|              | Wild (n, %)  | Altered (n, %) |         |         |
| **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 alteration and pathological markers in CRC

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.

**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).

**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.

**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).
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
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
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.

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.

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
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.

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