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ORIGINAL ARTICLE

Dickkopf-4 gene expression is associated with differentiation and lymph node metastasis in colorectal cancer

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Key words
colorectal cancer, Dickkopf-4, overall survival, Wnt/β catenin signal.

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Abstract

Background and Aim: Although high expression of Dickkopf-4 (DKK-4) in colorectal cancer has been reported in previous studies, its impact on clinicopathological features, including the prognosis and mechanism of expression, has not been well clarified to date.

Methods: (i) DKK-4 protein expression was analyzed by immunohistochemical staining with anti-DKK-4 antibody using archived formalin-fixed paraffin-embedded specimens obtained at surgery from 122 patients with colorectal cancer, and its association with clinicopathological findings was also investigated. (ii) The association between intratumoral DKK-4 protein expression and somatic hotspot mutations in cancer-associated genes in 40 patients was investigated using a next-generation sequencer.

Results: In cross-section, DKK-4 protein expression in colorectal tissue was related to an adenocarcinoma of a histologically differentiated type (tub1/tub2, $P = 0.032$) and distant metastasis ($P = 0.012$). Longitudinally, however, DKK-4 was not an independent prognostic factor determining overall survival (OS). On the other hand, in patients with metastasis, high DKK-4 protein was independently associated with short OS ($P = 0.013$). In addition, colorectal cancer tissue with high DKK-4 protein expression was associated with hotspot mutations in Wnt/β catenin-signaling molecules (APC, CTNNB1, and FBXW7, $P = 0.03$).

Conclusion: Intratumoral DKK-4 expression, by partly reflecting the Wnt/β catenin pathway, is strongly associated with the advancement of colorectal cancer and OS in colorectal cancer patients with metastasis, although further studies are needed.

Introduction

Colorectal cancer is the third most common cancer in men and the second in women worldwide[1]: 1.4 million patients are newly diagnosed, and 700 000 patients die annually. Although certain patients have benefited from chemotherapy, which includes conventional 5-fluorouril-based therapy, as well as recently developed monoclonal antibodies targeting vascular endothelial growth factor (VEGF) or epidermal growth factor receptor (EGFR) pathways, the pretreatment prediction of the response is difficult.[2] Under the circumstances, novel biomarkers enabling an early diagnosis or prediction of a chemotherapy response are required.[3,4]

The Dickkopf (DKK) family of genes encodes secreted proteins in vertebrates (DKK-1 to -4).[5–7] In general, DKK protein family members act as antagonists of Wnt proteins by binding to lipoprotein receptor-related protein 5/6 that induces binding complex endocytosis and inhibits Wnt/β catenin activation.[8–10] Recently, the DKK family was found to be involved in carcinogenesis in various organs; several studies report that DKK-1 or -3 are related to hepatocellular carcinoma, as well as gastric, ovarian, lung, or pancreatic cancers. With regard to DKK-1, several studies have examined the DKK-1 protein expression in patients with solid tumors through immunohistochemical staining (IHC) of tumor tissues or serum enzyme-linked immunoassay analysis and reported the close relationship between its expression and poor overall survival (OS).[11]

Previously, we found that DKK-4 was upregulated in colorectal cancer using clinical samples in a small number of patients and that activation of the Wnt/β catenin pathway induced the expression of DKK-4 through in vitro analysis, suggesting that DKK-4 expression might reflect activated Wnt/β catenin pathways in colorectal cancers.[12] DKK-4 was also reported to increase colon cancer cell migration and invasion to promote a proangiogenic phenotype, suggesting that DKK-4 itself has biological properties, enhancing malignant potential in colorectal cancer other than the mere reflection of activated Wnt/β catenin pathways.[13] From subsequent studies, it is also considered that DKK-4 expression may be involved in chemotherapy resistance in colorectal cancers.[14,15] However, clinical studies, to date, have not been undertaken on the role of the DKK-4 gene in its impact

on clinicopathological features, including prognosis and chemotherapy resistance, in patients with colorectal cancer.

In this study, we investigated the relationship between intratumoral DKK-4 protein expression and clinicopathological features through IHC analysis using formalin-fixed paraffin-embedded (FFPE) specimens of colorectal cancer with anti-DKK-4 antibody. We also examined the relationship between intratumoral DKK-4 protein expression and aberrations of cancer-related genes in colorectal cancer patients using a next-generation sequencer.

Methods

Tissue samples

Correlation between DKK-4 mRNA expression and protein expression of DKK-4. Before evaluating DKK-4 expression in archived colorectal cancer FFPE tissues with IHC staining, we investigated whether DKK-4 protein immunostaining really reflects *DKK-4 mRNA* expression in colorectal cancer tissues. To clarify this correlation, IHC staining was performed in 34 FFPE samples (17 colorectal carcinomas and paired normal tissues) in which mRNA expression in fresh frozen tissues had been previously investigated by real-time reverse transcription (RT)–polymerase chain reaction (PCR).[12]

Correlation between protein level of DKK-4 and clinicopathological features including prognosis. For DKK-4 immunostaining of colorectal cancers, archived FFPE samples were collected from 122 patients with colorectal cancer who had undergone a surgical resection at Yamanashi University (77 consecutive patients underwent a surgical resection from 2006 to 2007. The other 45 patients who had undergone a surgical resection were enrolled independent of the 77 patients: we had previously collected serum prior to surgery from 45 consenting consecutive patients with colorectal cancer between 2006 and 2012 for another study).

Correlation between cancer-related gene somatic mutations and intratumoral DKK-4 protein expression. For the analysis of the correlation between somatic mutation of cancer-related genes and DKK-4 protein expression in colorectal cancer, 40 FFPE tissues were randomly selected from the 122 FFPE tissues. Twenty FFPE samples, each showing high and low DKK-4 expression, were selected.

The study protocol conformed to the ethical guidelines of the 2000 Declaration of Helsinki, and consent was obtained from participants in the study, which was approved by the Human Ethics Review Committee of Yamanashi University.

Immunohistochemical staining. Anti-DKK-4 antibody (H-80; Santa Cruz Co., Heidelberg, Germany) was used as the primary antibody. IHC staining was performed according to the manufacturer's instructions. Briefly, deparaffinized sections of FFPE tissue at 3-μm thicknesses were stained with primary antibodies specific for DKK-4 (1:100 dilution; H-80; Santa Cruz Co.). Antigens were retrieved by boiling tissue sections in Target Retrieval Solution (Dako, Tokyo, Japan). Envision+Dual Link HRP (Dako) was used as the secondary antibody, and diaminobenzidine was used as the chromogen. IHC staining was blindly examined by two independent investigators. As a result, IHC intensities judged by two independent investigators were the same for most samples. However, a few samples with differing judgments were reexamined by the two investigators, and final results were determined after discussions.

Laser capture microdissection and DNA extraction. FFPE blocks were cut into 10 m-thick sections for laser microdissection and stained with HE. From FFPE specimens, tumor lesions and adjacent normal mucosa were separately microdissected using an ArcturusXT Laser Capture Microdissection System (Life Technologies, Carlsbad, CA, USA). DNA from resected tissues was extracted using a GeneRead DNA FFPE Kit (Qiagen, Milan, Italy) following the manufacturer's instructions. The quantity and quality of extracted DNA were assessed using a NanoDrop spectrophotometer (Thermo Fisher, Waltham, MA, USA) and a Qubit fluorometer (Thermo Fisher), respectively.

Targeted sequencing and variant calling. We used ready-made gene panels (Ion AmpliSeq Cancer Hotspot Panel v.2, Life Technologies) to amplify 50 cancer-related target genes. The panel contained 207 primer pairs and targeted 2790 hotspot mutations in the following 50 cancer-related genes in the COSMIC database 8: *ABL1*, *AKT1*, *ALK*, *APC*, *ATM*, *BRAF*, *CDH1*, *CDKN2A*, *CSF1R*, *CTNNB1*, *EGFR*, *ERBB2*, *ERBB4*, *EZH2*, *FBXW7*, *FGFR1*, *FGFR2*, *FGFR3*, *FLT3*, *GNA11*, *GNAS*, *GNAQ*, *HNF1A*, *HRAS*, *IDH1*, *JAK2*, *JAK3*, *IDH2*, *KDR/VEGFR2*, *KIT*, *KRAS*, *MET*, *MLH1*, *MPL*, *NOTCH1*, *NPM1*, *NRAS*, *PDGFRA*, *PIK3CA*, *PTEN*, *PTPN11*, *RB1*, *RET*, *SMAD4*, *SMARCB1*, *SMO*, *SRC*, *STK11*, *TP53*, and *VHL*. Briefly, 20 ng of DNA was amplified by PCR using such primer panels and an AmpliSeq HiFi Master Mix (Ion AmpliSeq Library Kit, Life Technologies). The multiplexed amplicons were treated with FuPa Reagent (Life Technologies) for the partial digestion of primer sequences and phosphorylation. The amplicons were then ligated to adapters from an Ion Xpress Barcode Adapters 1–96 Kit (Life Technologies) according to the manufacturer's instructions. After ligation, the amplicons underwent nick translation and additional library amplification by PCR to complete the linkage between adapters and amplicons. The size and concentration of the resultant amplicon libraries were then checked using a High Sensitivity DNA Kit (Agilent, Santa Clara, CA, USA) on an Agilent 2100 Bioanalyzer (Agilent) with on-chip electrophoresis.

Multiplexed barcoded libraries were amplified by emulsion PCR on Ion Sphere particles, and sequencing was performed on an Ion Chef System and Ion Proton Sequencer (Life Technologies) using an Ion PI Hi-Q Chef Kit (Life Technologies) according to the manufacturer's instructions. The Torrent Suite Software v.4.0 (Life Technologies) was used to align reads to an hg19 reference genome and generate run metrics, including total read counts and quality. A Variant Caller v.4.0 software plug-in (Life Technologies) was used to identify variants. The cut-off value of variant frequency per read depth was set at 5%.

Statistical analysis. The relationship between the expression level of *DKK-4 mRNA*, clinical background factor, mutation of a cancer-related gene, and immunostaining of DKK-4 was

examined by Chi-square test or Fisher's exact test. An analysis of survival was performed using the Kaplan–Meier method, a log-rank test was used for univariate analysis, and a Cox proportional hazards regression model was used for multivariate analysis. Two-tailed P values of <0.05 were considered to indicate statistical significance.

Results

DKK-4 mRNA expression correlated with DKK-4 protein expression by IHC. Initially, the intensity of IHC staining of DKK-4 was classified into four types[16]: 0+ (no staining at high magnification), 1+ (only visible at high magnification), 2+ (readily visible at low magnification), and 3+ (strikingly positive at low magnification, Fig. 1a).

In order to confirm whether DKK-4 protein expression by IHC staining reflected *DKK-4 mRNA* expression, IHC analysis was performed on FFPE samples in which *DKK-4 mRNA* expression had been investigated using fresh frozen tissues by real-time RT PCR in our previous report.[12] The relationship between the expression level of *DKK-4 mRNA* and protein IHC staining was examined.

DKK-4 protein expression in tumors and their adjacent normal mucosa was investigated (Fig. 1b). As shown, although DKK-4 staining was not observed in normal tissue, DKK-4 was stained to various degrees of intensity in tumors. Next, the correlation between *DKK-4 mRNA* expression and IHC staining intensity was investigated (Fig. 1c). It was shown that *DKK-4 mRNA* expression correlated with DKK-4 IHC intensity (P = 0.035, Fisher's exact test; Fig. 1c).

Colorectal cancers with high DKK-4 expression protein were associated with differentiated histological type and distant metastasis. The association

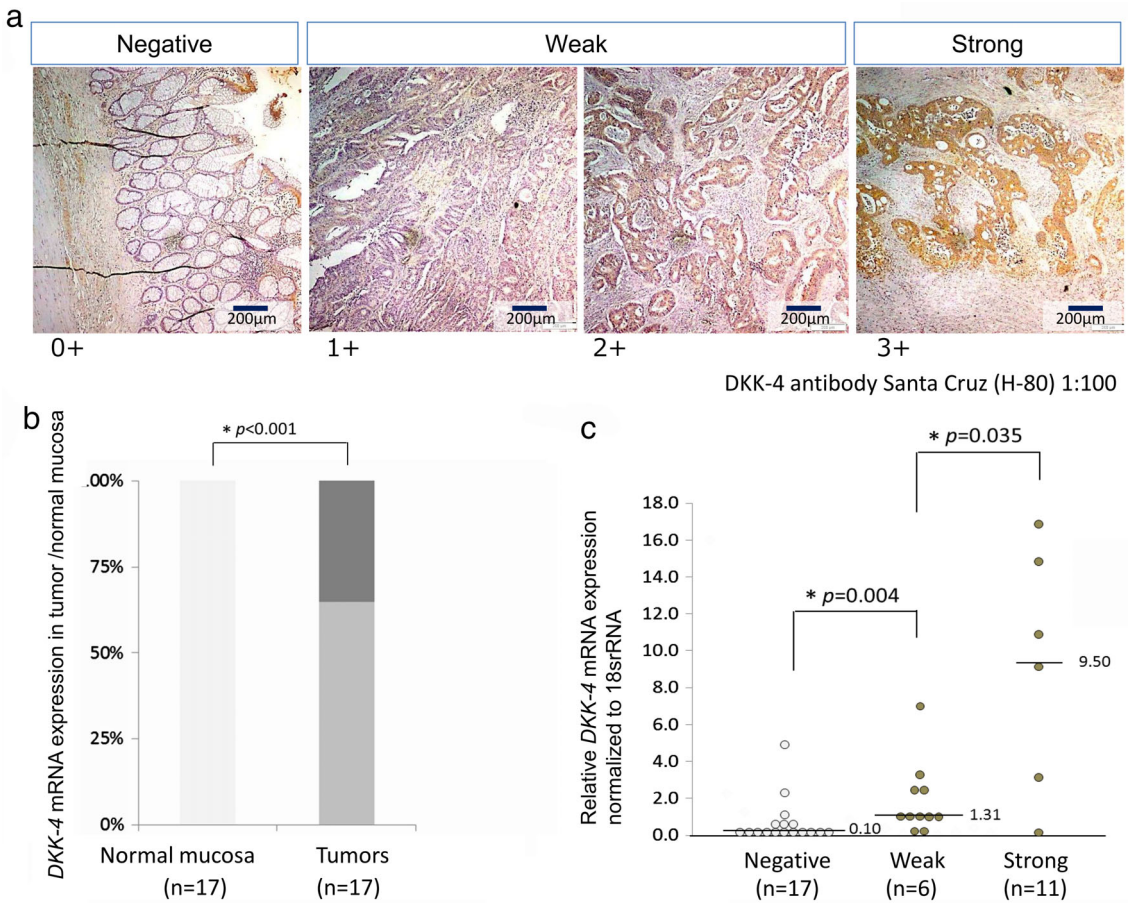

Figure 1 DKK-4 expression analyzed by real-time reverse transcription (RT)–polymerase chain reaction (PCR) was compared with that analyzed by immunohistochemical staining (IHC) in colorectal cancer tissues. (a) Immunohistochemical Dickkopf-4 (DKK-4) staining for colorectal cancer tissues (fresh frozen paraffin-embedded) was classified into four types according to its strength: 0+ (no staining at high magnification), 1+ (only visible at high magnification), 2+ (readily visible at low magnification), and 3+ (strikingly positive at low magnification). (b) DKK-4 expression analyzed by real-time (RT)–PCR was compared between normal mucosa and tumors. A stacked bar graph showing the intensity of *DKK-4* immunohistochemistry (IHC) expression. The intensity of DKK-4 expression was totally negative in normal mucosa. The intensity of DKK-4 expression in the tumor was either weak or strong: (■), strong; (▣), weak; (□), negative. (c) the correlation of *DKK-4 mRNA* expression with the intensity of DKK-4 protein IHC expression is shown in these scatter plots: (○), normal mucosa; (●), tumor.

Table 1 Clinicopathological characteristics of patients

Clinicopathological characteristics	Total (n = 122)	DKK-4 weak (n = 91)	DKK-4 strong (n = 31)	OR (95% CI)	P value
Age, no. (%)					
<65 years	54 (44.3)	40	14	0.95 (0.42–2.16)	1
≧65 years	68 (55.7)	51	17		
Gender, no. (%)					
Male	79 (64.8)	58	21	0.84 (0.35–1.99)	0.828
Female	43 (35.2)	33	10		
TNM staging, no. (%)					
I/II	61 (50.0)	48	13	1.55 (0.68–3.52)	0.405
III/IV	61 (50.0)	43	18		
Tumor invasion, no. (%)					
T1–T3	91 (74.6)	71	20	1.95 (0.80–4.74)	0.155
T4	31 (25.4)	20	11		
Lymph nodes involved, no. (%)					
N0	63 (51.6)	50	13	1.69 (0.74–3.85)	0.22
N1/N2	59 (48.4)	41	18		
Metastasis status, no. (%)					
M0	86 (70.5)	70	16	3.13 (1.33–7.36)	0.012†
M1	36 (29.5)	21	15		
Location, no.(%)					
Left-sided (R/S/D)	75 (61.5)	55	20	0.84 (0.36–1.96)	0.831
Right-sided (T/A/C)	47 (38.5)	36	11		
Histological type, no. (%)					
Tub1/Tub2	110 (90.2)	79	31	—	0.032†
Por/Muc/others	12 (9.8)	12	0		

†χ^2 test.

A, ascending colon; C, Cecum; CI, confidence interval; D, descending colon; Dickkopf-4, DKK-4; Muc, mucinous adenocarcinoma; OR, odds ratio; Por, poorly differentiated adenocarcinoma; R, rectum; S, sigmoid colon; T, transverse colon; TNM, T, primary tumor; N, regional lymph nodes; M, distant metastasis; tub1, well-differentiated adenocarcinoma; tub2, moderately differentiated adenocarcinoma.

between DKK-4 IHC staining intensity and clinical features was investigated in a cross-sectional manner by dividing patients into two groups according to DKK-4 IHC density. Of 122 patients, 31 (25%) and 91 patients (75%) were classified into strong and weak groups, respectively (Table 1). As shown here, clinicopathological characteristics of histologically differentiated adenocarcinomas (P = 0.032) and metastases (P = 0.012) were significantly more frequently noted in the DKK-4 strong group (Table 1). Interestingly, all tumor tissues were stained with DKK-4 to some degrees (weak to strong), and no tissues were negative on IHC staining.

Colorectal cancers with high DKK-4 protein expression were associated with a shorter OS in patients with metastasis. The association between DKK-4 IHC staining intensity and OS was analyzed by Kaplan–Meier curve analysis. The median observation period was 36.6 months. As shown in Figure 2, OS in patients with strong IHC staining was significantly shorter than that in patients with weak staining as found by log–rank analysis (P = 0.0012; Fig. 2). However, multivariate analysis using a Cox regression model for clinical characteristics affecting OS demonstrated that the presence of lymph node metastases and distant metastases were independent prognostic factors contributing to survival. Although DKK-4 IHC staining intensity was associated with OS in univariate analysis, the association did not reach significance in multivariate analysis (Table 2).

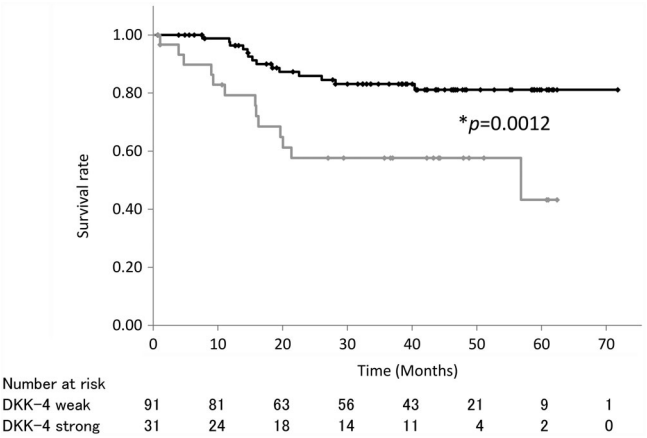

Figure 2 The influence of Dickkopf-4 (DKK-4) expression on overall survival in patients with colorectal cancer is demonstrated in a Kaplan–Meier curve. Patients who showed strong DKK-4 (strong) staining in immunohistochemical staining compared to those who showed weak DKK-4 staining (weak) had a lower survival rate. *Log-rank test. (——), DKK-4 weak; (——), DKK-4 strong.

Although DKK-4 was not an independent factor determining OS when all patients were included, we undertook subanalyses to find a further role for DKK-4 in specific populations. Namely, we divided patients according to metastasis (M) status,

Table 2 Results of the multivariate analysis associated with overall survival

Clinicopathological characteristics	Univariate analysis *P* value	Multivariate analysis HR	95% CI	*P* value
Age (years), <65 *vs* ≧65	0.181	–	–	–
Gender, male *vs* female	0.094	–	–	–
Tumor invasion, T1–3 *vs* T4	0.032*	1.56	0.63–3.85	0.333
Lymph nodes involved, N0 *vs* N1/N2	<0.001**	5.43	1.05–28.08	0.044*
Metastasis status, M0 *vs* M1	<0.001**	5.84	1.86–18.34	<0.001**
Location, left-sided *vs* right-sided	0.831	–	–	–
Histological type, Tub1/Tub2 *vs* Por/Muc/others	0.995	–	–	–
DKK-4, weak *vs* strong	0.001**	1.88	0.80–4.42	0.146

Univariate analysis: log-rank test. Multivariate analysis: Cox regression analysis.
CI, confidence interval; Dickkopf-4, DKK-4; HR, hazard ratio; M, distant metastasis; Muc, mucinous adenocarcinoma; N, regional lymph nodes; Por, poorly differentiated adenocarcinoma; T, primary tumor; tub1, well-differentiated adenocarcinoma; tub2, moderately differentiated adenocarcinoma.* *P* value <0.05, ** *P* value < 0.01.

which was an independent factor for OS, and investigated the influence of DKK-4 IHC intensity on OS. As shown in Table 3, DKK-4 IHC staining intensity was an independent factor determining OS in the multivariate analysis of M1 patients ($P = 0.033$). When all patients were divided according to M status, the OS of M1 patients was shorter than that of M0 patients ($P < 0.001$; Fig. 3a). In subgroup analysis, although DKK-4 IHC staining intensity did not influence OS in the M0 group ($P = 0.268$; Fig. 3b), those with a strong DKK-4 IHC staining intensity had a shorter OS than those with a weak DKK-4 IHC staining intensity in the M1 group ($P = 0.013$; Fig. 3c).

DKK-4 protein expression by IHC is associated with mutations in genes inducing Wnt signaling. To investigate the correlation between DKK-4 protein IHC expression in colorectal cancer and somatic mutation of cancer-related genes, next-generation sequencing targeting cancer-related genes using a ready-made gene panel (Ion AmpliSeq Cancer Hotspot Panel v.2, Life Technologies) was performed for several FFPE samples. Forty FFPE tissues were randomly selected from 122 FFPE tissues: 20 were randomly selected from FFPE samples showing high DKK-4 protein expression, and the other 20 samples were randomly selected from those with low DKK-4 protein expression.

As shown in Figure 4, hotspot mutations were found in 14 different genes, with a *TP53* mutation being the most frequent

Table 3 Results of the multivariate analysis associated with overall survival in M1 patients (n = 36)

Clinicopathological characteristics	Univariate analysis *P* value	Multivariate analysis HR	95% CI	*P* value
Age (years), <65 *vs* ≧65	0.606	–	–	–
Gender, male *vs* female	0.480	–	–	–
Tumor invasion, T1–3 *vs* T4	0.104	1.69	0.71–4.01	0.234
Lymph nodes involved, N0 *vs* N1/N2	0.592	–	–	–
Location, left-sided *vs* right-sided	0.576	–	–	–
Histological type, Tub1/Tub2 *vs* Por/Muc/others	0.716	–	–	–
DKK-4, weak *vs* strong	0.0127*	2.57	1.08–6.12	0.033*

Univariate analysis: log-rank test. Multivariate analysis: Cox regression analysis.
CI, confidence interval; Dickkopf-4, DKK-4; HR, hazard ratio; M, distant metastasis; Muc, mucinous adenocarcinoma; N, regional lymph nodes; Por, poorly differentiated adenocarcinoma; T, primary tumor; tub1, well-differentiated adenocarcinoma; tub2, moderately differentiated adenocarcinoma.* *P* value <0.05, ** *P* value < 0.01

(72.5%, 29/40; Fig. 4). As DKK family molecules are induced by Wnt signaling, we next focused our analysis on genes in the Wnt signaling pathway. We found that 42.5% (17/40) of cancers had at least one hotspot mutation in *APC*, *FBXW7,* or *CTNNB1* genes, which are located in the Wnt signaling pathway (Fig. 4).

Next, we investigated the relationship between DKK-4 IHC staining intensity and somatic gene mutations in the Wnt signaling pathway. As shown in Figure S1, Supporting information, hotspot mutations in *APC*, *FBXW7,* or *CTNNB1* genes were not found among cancers with weak IHC intensity (1+), while many mutations existed in these genes among cancers with a strong IHC intensity (2+ and 3+; $P = 0.030$).

Association of DKK-4 protein expression with response to chemotherapy in patients with distant metastasis. Finally, we investigated whether DKK-4 IHC staining intensity contributes to the response to 5-FU-based chemotherapy in patients with advanced disease (distant metastasis). There was a marginal association between patients with high DKK-4 protein expression and a poor response when initial treatment was evaluated ($P = 0.089$, Fig. S2).

Discussion

In this study, using archived FFPE specimens obtained at surgery for colorectal cancers, we found that high DKK-4 protein expression in tumor tissue was associated with histologically differentiated types and distant metastases in a cross-sectional analysis. Longitudinally, high intratumoral DKK-4 protein expression was associated with short OS among advanced stage patients due to distant metastasis or lymph node metastasis. In investigating the correlation with cancer-related genes, high DKK-4 protein

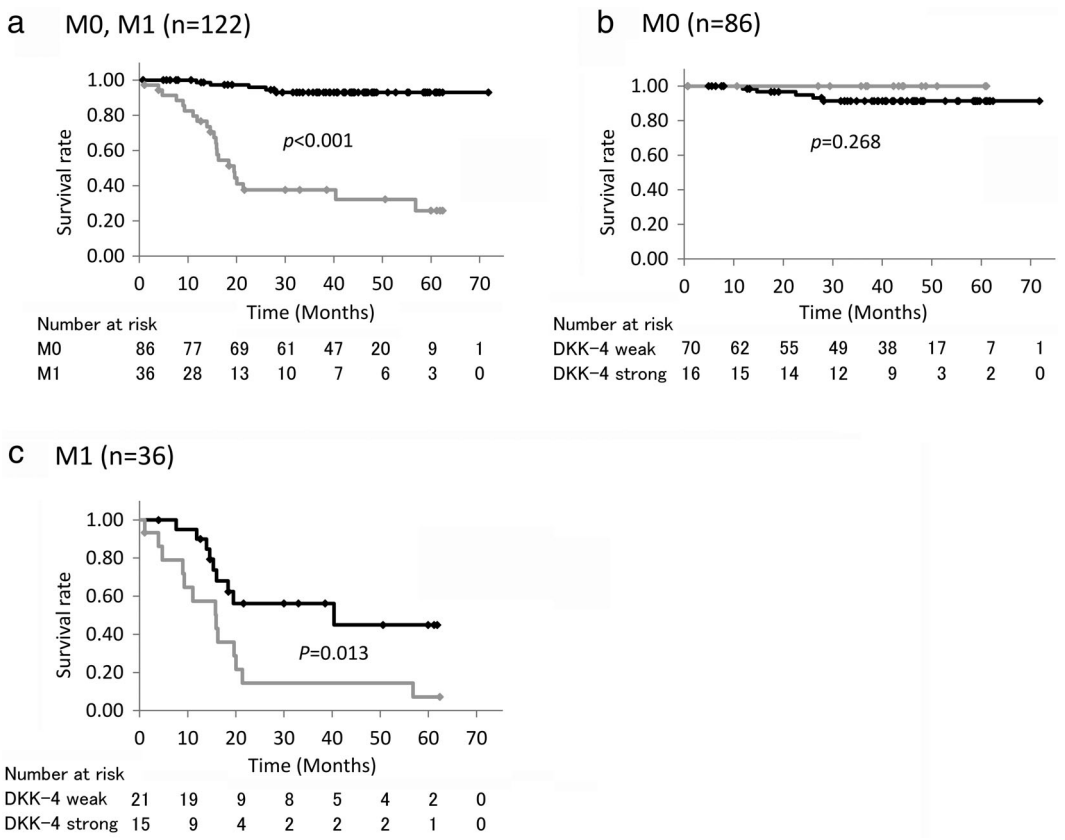

Figure 3 The influence of Dickkopf-4 (DKK-4) expression on overall survival (OS) in patients divided by M factor is demonstrated. (a) OS in patients divided by M factor is demonstrated: (——), M0; (——), M1. (b) OS in M0 patients divided by DKK-4 expression is demonstrated: (——), DKK-4 weak; (——), DKK-4 strong. (c) OS in M1 patients divided by DKK-4 expression is demonstrated: (——), DKK-4 weak; (——), DKK-4 strong.

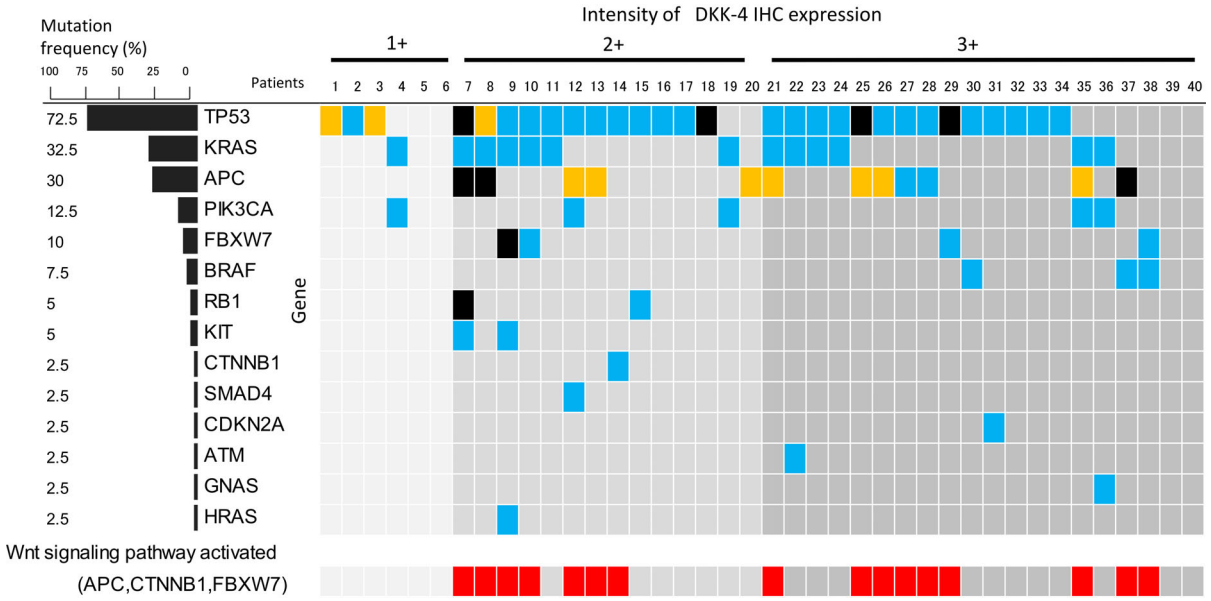

Figure 4 The association between Dickkopf-4 expression and hotspot somatic mutations in cancer-related genes is demonstrated. (■), Missense mutation; (■), frameshift indel; (■), nonsense mutation. IHC, immunohistochemical staining.

expression was associated with somatic gene mutations in Wnt/β catenin pathway molecules.

To date, in human cancers, high expression of DKK-4 has been observed in ovarian, pancreatic, and renal cancers. An association between high DKK-4 expression and short OS has been reported in renal and ovarian cancers. In colorectal cancers, although we had previously demonstrated increased expression of *DKK-4 mRNA* in a small number of patients, we have confirmed its high expression in a larger cohort, to a varying degree, through protein analysis. We also demonstrated a positive correlation between expression of mRNA and that of protein (Fig. 1). On the other hand, there was a discrepancy between DKK-4 protein and mRNA in some patients: several cancers with high DKK-4 protein showed low mRNA (Fig. 1). Although the reason is not clear, analyzed tissue size was different between the two methods. While small biopsy samples obtained at endoscopy before surgical resection were used in mRNA analysis, large surgically resected tissues were used in IHC analysis. As DKK-4 expression was rather heterogeneous depending on cancer tissues, small biopsy samples might not always reflect the whole cancer tissues, and further studies are warranted. Moreover, we investigated and demonstrated clinicopathological features in colorectal cancers associated with strong/weak DKK-4 expression in this study.

Why does intratumoral DKK-4 protein expression reflect malignant characteristics of colorectal cancers? As DKK-4 is one of the target molecules regulated by activation of the Wnt/β catenin-signaling pathway, which induces multiple downstream genes promoting tumor development,[12,17] it is possible that DKK-4 expression merely reflects the activated state of Wnt/β catenin signaling and that DKK-4 acts as a surrogate for the Wnt/β catenin signal. Wnt/β catenin signaling is a major pathway in the development of colorectal cancer and is activated through somatic mutations of pathway molecules, such as APC, CTNNB1, TCF7L2, or FBXW7 proteins within cancerous tissues.[18,19] We found a correlation of DKK-4 protein expression in colorectal cancer tissues with mutations of *APC*, *CTNNB1,* and *FBXW7* genes in this study (Fig. S1). However, high DKK-4 protein expression was also observed in cancers that did not have somatic mutations in *APC*, *CTNNB1,* and *FBXW7* genes in this study, suggesting that factors other than Wnt/β catenin signaling may contribute to high DKK-4 expression (Fig. S1). It has recently been found that the expression of TFAP2E protein, a transcriptional factor, is elevated in colorectal cancer with high susceptibility to fluorouracil-based chemotherapy and that DKK-4 may be a possible downstream target protein determining the susceptibility downregulated by TFAP2E protein.[14] As TFAP2E-induced DKK-4 expression is considered independent of the Wnt/β catenin-signaling pathway, the TFAP2E pathway may be another pathway in our highly expressing DKK-4 colorectal cancers that do not have mutations in Wnt/β catenin pathway molecul, although further studies are needed to verify this hypothesis. In this study, however, the *APC* mutation rate was low (30%), while the *TP53* mutation rate was high (72.5%) when compared with previous analysis (44 and 43% in COSMIC database, respectively). Although the reason is unclear, it is possible that the coverage of hotspot mutations with the cancer panel used in this study might have some biases according to each gene as a previous study using the same panel also reported low *APC* (36%) and high *TP53* (65%) mutation rates in colorectal cancer.[20]

Moreover, why was DKK-4 protein expression associated with distant metastases and a well-differentiated histological type? As stated earlier, it is possible that such features of cancer may simply reflect the status of Wnt/β catenin signal activation that promotes tumor progression. However, according to previous in vitro studies, it is also possible that the DKK-4 protein itself functions as a molecule to enhance tumor progression. In renal, ovarian, and pancreatic cancers, DKK-4 activates the Wnt non-canonical pathway and is involved in tumor proliferation via the MAPK signal transduction pathway.[21,22] In a colorectal cancer cell line, DKK-4 itself promotes invasiveness and angiogenesis, although the mechanism of action is still undetermined.[17,23] Such capacities for invasion and angiogenesis are compatible with the high frequency of distant metastases in high DKK-4-expressing tumors, although the association with a well-differentiated histological type is still unclear. DKK-4 itself may become a target of anticolorectal cancer therapy when it enhances tumor progression; however, further in vitro as well as in vivo studies are required.

Why are high DKK-4-expressing tumors associated with a poor prognosis in advanced colorectal cancer patients with distant metastases or lymph node metastases? As stated earlier, DKK-4 has been assumed to have a role in the resistance to fluorouracil-based chemotherapy from the clinical observation that TFAP2E was associated with a fluorouracil-based chemotherapy response in colorectal cancer.[14] In colon cancer cell lines expressing high levels of DKK-4 protein, the sensitivity to 5-fluorouracil was decreased.[15] In this study, when the association of DKK-4 expression with the patient's response to fluorouracil-based chemotherapy was investigated, the outcome tended to be more favorable for those with low DKK-4 protein expression (Fig. S2), supporting the previous hypothesis. Although further studies are still needed to confirm the hypothesis, the result indicates that DKK-4 expression could be a biomarker predicting OS, possibly through the control of chemotherapy resistance. Furthermore, if the above hypothesis is true, DKK-4 protein may also be a new target of anticolorectal cancer therapy by enhancing a patient's susceptibility to chemotherapy.

In conclusion, we demonstrated the following points: (i) Intratumoral DKK-4 protein expression in surgically resected colorectal cancers was upregulated in most cases to various degrees. (ii) High DKK-4 protein expression was associated with a well-differentiated histological type, the presence of distant metastases, and poor OS. (iii) High intratumoral DKK-4 protein expression was associated with somatic mutations in molecules of the Wnt/β catenin-signaling pathway. It is possible that further DKK-4 studies might help clarify the mechanisms of colorectal cancers and their therapeutic molecular targets.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's website:

Figure S1 The association between DKK-4 expression and mutations in Wnt/β catenin-signaling molecules (APC, CTNNB1, and FBXW7) is demonstrated.
Figure S2 The correlation between DKK-4 IHC staining intensity and the initial response to 5-fluoruracil (FU)–based chemotherapy was investigated in patients with distant metastasis. A partial response (PR) and stable disease (SD) were classified as beneficial responses, while progressive disease (PD) was classified as a poor response.