Table 4 Association of single nucleotide polymorphisms (SNPs) at 4q23 and 12q24 with oesophageal squamous cell carcinoma (OSCC) in the Japanese samples

SNP (Chromosome; position*)	Screening stage†	Risk allele frequency		OR (95% CI)‡	p Value‡	Genotyping success rate (%)	
		OSCC	Control			OSCC	Control
rs3805322	First	0.45	0.38	1.35 (1.09 to 1.66)	0.0056	100	100
(Chr.4; 100276021)	Second	0.47	0.39	1.42 (1.21 to 1.67)	1.5×10^{-5}	100	100
	Third	0.47	0.35	1.65 (1.38 to 1.97)	4.1×10^{-8}	100	100
	Combined	0.47	0.37	1.48 (1.34 to 1.64)	4.5×10^{-14}	100	100
rs1042026	First	0.22	0.16	1.47 (1.13 to 1.90)	0.0038	100	99.6
(Chr.4; 100447489)	Second	0.26	0.18	1.53 (1.26 to 1.85)	1.0×10^{-5}	99.8	100
	Third	0.26	0.16	1.83 (1.48 to 2.26)	1.9×10^{-8}	99.7	99.9
	Combined	0.25	0.17	1.66 (1.47 to 1.88)	1.8×10^{-16}	99.8	99.8
rs2238149	First	0.30	0.19	1.79 (1.41-2.27)	1.1×10^{-6}	99.0	98.2
(Chr.12; 109796312)	Second	0.26	0.21	1.32 (1.10 to 1.59)	0.0031	100	100
	Third	0.24	0.20	1.26 (1.02 to 1.56)	0.032	100	99.9
	Combined	0.26	0.20	1.41 (1.25 to 1.58)	1.3×10^{-8}	99.8	99.3
rs11065756	First	0.34	0.21	1.92 (1.53 to 2.42)	1.2×10^{-8}	99.5	100
(Chr.12; 109823177)	Second	0.30	0.23	1.43 (1.20 to 1.71)	7.1×10^{-5}	100	100
	Third	0.27	0.22	1.26 (1.03 to 1.54)	0.026	100	99.9
	Combined	0.30	0.22	1.48 (1.33 to 1.66)	7.1×10^{-12}	99.9	100
rs11065783	First	0.40	0.30	1.56 (1.26 to 1.94)	4.9×10^{-5}	100	100
(Chr.12; 109880632)	Second	0.37	0.31	1.35 (1.14 to 1.59)	0.00046	100	100
	Third	0.33	0.28	1.24 (1.02 to 1.50)	0.029	99.7	100
	Combined	0.36	0.29	1.35 (1.21 to 1.50)	3.1×10^{-8}	99.9	100
rs12229654	First	0.34	0.21	1.99 (1.59 to 2.50)	2.1×10^{-9}	99.5	99.8
(Chr.12; 109898844)	Second	0.30	0.21	1.61 (1.35 to 1.93)	1.9×10^{-7}	100	100
	Third	0.27	0.20	1.54 (1.26 to 1.89)	3.4×10^{-5}	99.7	100
	Combined	0.30	0.20	1.66 (1.48 to 1.86)	3.3×10^{-18}	99.8	99.9
rs2074356	First	0.36	0.22	1.97 (1.57 to -2.46)	2.2×10^{-9}	100	100
(Chr.12; 111129784)	Second	0.32	0.23	1.53 (1.29 to 1.83)	1.7×10^{-6}	100	100
	Third	0.32	0.21	1.77 (1.46 to 2.16)	1.1×10^{-8}	100	100
	Combined	0.33	0.22	1.70 (1.52 to 1.90)	3.9×10^{-21}	100	100
rs11066280	First	0.40	0.26	1.92 (1.54 to 2.39)	3.2×10^{-9}	100	99.1
(Chr.12; 111302166)	Second	0.37	0.27	1.52 (1.29 to 1.81)	8.9×10^{-7}	100	100
	Third	0.36	0.25	1.71 (1.41 to 2.06)	2.8×10^{-8}	99.7	99.7
	Combined	0.37	0.26	1.68 (1.51 to 1.87)	2.5×10^{-21}	99.9	99.6

*SNP position is based on NCBI build 36.
†The number of samples at each stage was as follows: 209 and 1079 in the first stage, 479 and 863 in the second stage, and 365 and 780 in the third stage for OSCC and controls, respectively.
‡The odds ratio and p value were calculated by an allele test.

which reduce enzymatic activity (table 5). The frequency of the GG genotype of rs1229984 was higher in OSCC than in controls (0.20 vs. 0.06, OR=4.08, $p=4.4\times10^{-40}$). Similarly, the frequency of the AA+AG genotype of rs671 was higher in cases than in controls (0.73 vs 0.43, OR = 3.54, $p=5.5\times10^{-62}$). These results indicate that individuals who exhibit low enzymatic activity for *ADH1B* and/or *ALDH2* are at higher risk for OSCC.

Table 2 shows the ORs of OSCC associated with environmental and genetic risk factors along with their internal interactions. Ever-drinkers who did not smoke and ever-smokers who did not drink alcohol had significantly elevated adjusted ORs of 3.5 (95% CI 2.1 to 5.8) and 2.3 (95% CI 1.2 to 4.3), respectively. A supra-multiplicative OR of 16.0 (95% CI 9.7 to 26.3)—that is, statistically larger than the product of 3.5 and 2.3 (8.0), was found among individuals who were both ever-drinkers and ever-smokers. Subjects with only one risk allele, either rs671 AG/AA or rs1229984 GG, had significantly higher ORs of 4.8 (95% CI 3.7 to 6.3) and 3.1 (95% CI 1.6 to 6.1), respectively, than those without either risk allele. The OR for those with both genetic risk factors was 34.0 (95% CI 18.1 to 63.8); however, the interaction of these two genetic factors did not reach significance (p=0.079).

We also evaluated the combined effects of environmental and genetic risk factors (table 3). In this analysis, the reference group was composed of individuals who never drank or smoked and who also had no genetic risk factors. Compared with the reference group, ever-drinkers who did not smoke and had no genetic risk factors had a non-significant OR of 1.5 (95% CI 0.7 to 3.3). Non-drinkers and non-smokers with genetic risk factors also had a non-significant OR of 1.1 (95% CI 0.5 to 2.4). All other groups, however, had significantly elevated ORs. An interaction between alcohol drinking and smoking was observed only in the stratum with genetic risk. In the stratum with no genetic risk factors, alcohol drinking was not associated with OSCC, regardless of smoking status. Smoking without alcohol drinking elevated the ORs, regardless of the rs671 and rs1229984 genotypes, similarly and significantly. In contrast, interactions between alcohol drinking and genetic risk factors were highly significant. The combined effects of alcohol drinking and genetic risk factors were larger than the products of individual effects. For example, among non-smokers, the combined OR (12.1) was significantly larger than the product of the genetic effect (1.1) and the alcohol drinking risk (1.5). The same effect was seen among smokers (62.1>1.1×5.0).

Finally, we evaluated the effect on OSCC of the number of risk factors present of the two possible environmental and two possible genetic factors (Figure 1). Compared with the no-risk-factor condition, the ORs for one, two, three and four risk factors were 1.4 (95% CI 0.7 to 2.7), 4.3 (95% CI 2.2 to 8.4), 41.0

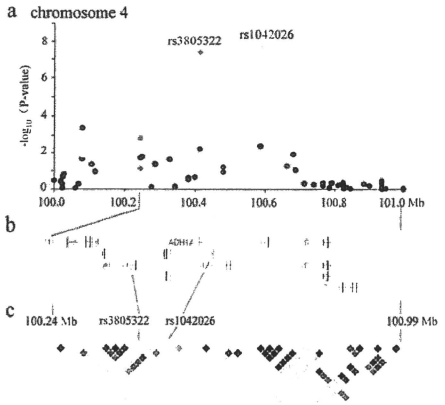

Figure 3 The 4q23 locus is associated with oesophageal squamous cell carcinoma (OSCC). (A) Single nucleotide polymorphism (SNP) single-marker association results. All genotyped SNPs at this locus in this study are plotted with their $-\log_{10}$(p value) for OSCC (allelic test) against chromosome position in Mb (100.0 to 101.0). Black, green and red dots indicate p values at the first, second and third screening stages. Two highly significant SNPs from the combined analysis, rs380532 and rs1042026, are shown. (B) The genomic location of RefSeq genes (100.24–100.99 Mb) with intron and exon structures is shown (UCSC Genome Browser on Human Mar. 2006 Assembly). (C) Pairwise square of the correlation coefficient (r^2) estimates for 39 SNPs from 100.24 Mb to 100.99 Mb in controls at the first stage, with increasing shades of grey indicating higher r^2 values.

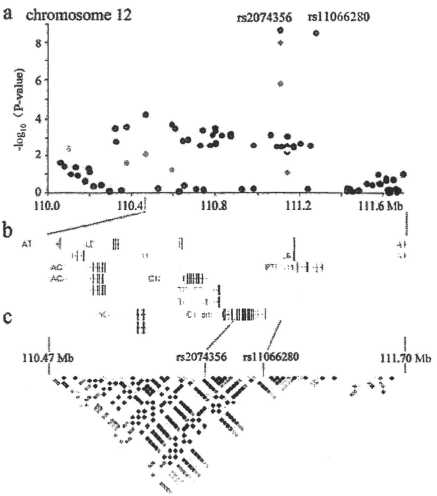

Figure 4 The 12q24.11-13 locus is associated with oesophageal squamous cell carcinoma (OSCC). (A) Single nucleotide polymorphism (SNP) single-marker association results. All SNPs genotyped at this locus in this study are plotted with their $-\log_{10}$(p value) for OSCC (allelic test) against chromosome position in Mb (110.0–111.7). Black, green and red dots indicate p values at the first, second and third screening stages. Two highly significant SNPs in the combined analysis, rs2074356 and rs11066280, are shown. (B) The genomic location of RefSeq genes (110.47–111.70 Mb) with intron and exon structure is shown (UCSC Genome Browser on Human Mar. 2006 Assembly). (C) Pairwise square of the correlation coefficient (r^2) estimates for 63 SNPs from 110.47 Mb to 111.70 Mb in controls at the first stage, with increasing shades of grey indicating higher r^2 values.

(95% CI 20.2 to 83.3) and 357.1 (95% CI 105.4 to 1209.5), respectively. A highly significant linear trend ($p<0.0001$) was observed.

DISCUSSION

Individuals who smoke and drink alcohol are considered at high risk for OSCC, although most such people do not develop the disease. Indeed, in a recent study, only 41 of 100 000 such people developed OSCC.[9] Therefore, it is crucial to simultaneously analyse genetic and environmental risk factors to more efficiently identify people at truly high risk for OSCC. This unbiased genome-wide association study identified two loci

Table 5 Association of non-synonymous single nucleotide polymorphisms (SNPs) in *ADH1B* and *ALDH2* with oesophageal squamous cell carcinoma (OSCC)

SNP (Chromosome; gene)	Study group*	Genotype frequency†			Allele frequency		HWE test p Value	Genotype association test‡ OR (95% CI)	p Value	Allele association test OR (95% CI)	p Value	PAR§ (%)
		GG	GA	AA	G	A						
rs1229984	OSCC	0.20	0.32	0.48	0.36	0.64	9.4×10^{-23}	4.08 (3.27 to 5.09)	4.4×10^{-40}	1.82 (1.63 to 2.03)	6.23×10^{-28}	41.5
(Chr.4; *ADH1B*)	Control	0.06	0.35	0.59	0.23	0.77	0.56					
		AA	AG	GG	A	G						
rs671	OSCC	0.01	0.72	0.27	0.37	0.63	1.7×10^{-81}	3.54 (3.04 to 4.14)	5.5×10^{-62}	1.78 (1.60 to 1.98)	1.03×10^{-26}	38.8
(Chr.12; *ALDH2*)	Control	0.07	0.36	0.57	0.25	0.75	0.12					

The risk allele of rs1229984 (G) and rs671 (A) encode arginine at position 48 and lysine at position 504, respectively, which are known to reduce enzymatic activity.
HWE, Hardy–Weinberg equilibrium.
*The number of OSCC and control samples was 1071 and 2762, respectively.
†The genotyping success rate of rs1229984 and rs671 was 99.84% and 99.79%, respectively.
‡A genotype association analysis was performed for GG versus GA+AA genotype in rs1229984 and for AA+AG versus GG genotype in rs671.
§The population attributable risk (PAR = AF*(OR − 1)/(AF*(OR − 1) + 1)) (AF, allele frequency) is defined as the reduction in the incidence of the disease if the population were not exposed to the risk allele.

containing genes involved in alcohol metabolism. In addition, we found a strong genetic-environmental interaction related to the risk of OSCC. Subjects with two environmental risk factors (ever-smokers and ever-drinkers) in combination with two genetic risk factors (AA or GA at rs1229984 (*ADH1B*) and GG at rs671 (*ALDH2*)) had a much higher risk than other subjects. Specifically, compared with no risk factor, the ORs with one, two, three and four risk factors were 1.4 (95% CI 0.7 to 2.7), 4.3 (95% CI 2.2 to 8.4), 41.0 (95% CI 20.2 to 83.3), and 357.1 (95% CI 105.4 to 1209.5), respectively. Of all of the risk factors for OSCC that we had previously examined, the combination of all four factors studied had the highest risk, with an OR of 357.1.

This information on the strong genetic-environmental interaction is valuable for secondary prevention of OSCC. When we see subjects who show *ADH1B* (rs1229984) and *ALDH2* (rs671) variants as well as smoking and drinking habits, we will advise them to have a periodic upper gastrointestinal fibre test. Screening of these patients could have an important role in the early detection of OSCC. Furthermore, the information gained from this study may also enable the development of a primary individualised prevention strategy for young people with these genetic variations. When subjects have these high-risk variants, advising them not to start smoking or, especially, not to drink alcohol will dramatically reduce their risk of developing OSCC. We observed that drinkers who consumed alcohol daily and heavy smokers had higher ORs than their counterparts, and the ORs decreased with an increased amount of time since quitting these habits. However, all ORs among ever-drinkers/smokers were significantly higher than 1.0 (supplementary table 2). At present, we cannot unequivocally determine the preventive effect of quitting smoking or drinking alcohol. To determine whether stopping these habits reduces the risk of OSCC, prospective studies are needed.

Several limitations of this study should be mentioned. First, the statistical power of this genome-wide association study was not sufficient for allelic variants with an OR of <1.5 (supplementary table 1). Therefore, we might have missed variants with a small effect size (eg, 1.1–1.3), which are often reported for other life-style-related diseases. Second, we did not match the cases and controls; thus, the basic distributions of sex, place of residence and age were different between the two groups (table 1). However, although matching is efficient in data collection, it does not affect the point estimation if the factor is included in the model. Thus, the absence of matching did not distort the results. Third, personalised genetic testing is prohibitively expensive and ethically problematic. Finally, because of the retrospective study design, several answers in the questionnaire could be altered by disease or pre-disease conditions. Thus, the high OR among former drinkers and smokers who had quit less than 1 year previously may incorrectly imply a causal relationship between these habits and OSCC risk. Therefore, the effect of quitting these habits on OSCC risk should be examined by prospective studies.

Hashibe *et al* identified the variation of *ADH1B* (rs1229984) as a risk factor for OSCC.[10] They conducted their analysis on European and Latin American populations, and the result was consistent with the results of our study of Japanese patients and controls. A study of Chinese people also demonstrated that *ADH1B* (rs1229984) is a risk factor for OSCC.[11] Recently, Cui *et al* reported that variations of *ADH1B* (rs1229984) and *ALDH2* (rs671) coupled with alcohol drinking and smoking synergistically enhance oesophageal cancer risk.[12] Their study indicates that these two genetic risk variants provide almost equal risk for the generation of OSCC. In our study, among individuals without a genetic risk, alcohol consumption did not increase the OR of OSCC significantly. For people without genetic risk, smoking habits were the major contributing factor for the generation of OSCC. However, in people with genetic risk, a drinking habit strikingly increased the risk of OSCC; combined with smoking, it increased the risk even further (table 3).

Smoking- and alcohol drinking-related genes such as the *ADH* family, the *ALDH* family and nicotinic acetylcholine receptors, especially as indicated in SNP analyses, have been significantly associated with a variety of cancers[10 13–15] We performed an association analysis between OSCC and SNPs in the nicotinic acetylcholine receptor subunit genes *CHRNA3* and *CHRNA5* at 15q25 using the same cases and controls. An association with lung cancer was reported by Hung *et al*[14] and Thorgeirsson et *al*[13] However, in this study, we did not find a significant association at either rs1051730 (*CHRNA3*) or rs16969968 (*CHRNA5*), with ORs of 0.91 and 0.89, respectively (supplementary table 3). Smoking habits contributed to the development of OSCC; however, SNPs other than rs1051730 (*CHRNA3*) or rs16969968 (*CHRNA5*) might affect OSCC generation. Because the risk allele frequency of both of these SNPs was <0.02 in cases and controls in this study population, it would be difficult to show any difference between cases and controls. Variants of *ECRG1* have been reported to be associated with OSCC.[16] However, it is still unclear whether the genetic or epigenetic changes caused by smoking and/or alcohol drinking are directly associated with the development of OSCC in cooperation with SNPs of genes such as those of the ADH family, the ALDH family and nicotinic acetylcholine receptors[5 17] ADH1B in subjects with the rs1229984 AA or GA genotype is reported to metabolise ethanol up to 40 times more quickly than ADH1B from GG homozygotes.[18] Furthermore, ALDH2 from subjects with the GG allele of rs671 is reported to metabolise acetaldehyde more than 100 times faster than from AG ALDH2 heterozygotes.[19] In addition, *ADH* genes exhibit a nominally significant association with smoking behaviour.[20] Considering our results and these reports, higher local exposure to ethanol and acetaldehyde mediated by smoking may be strongly associated with OSCC development. To answer these questions, it is necessary to conduct a prospective study in genetically at-risk populations with or without drinking and/or smoking habits, as recently performed for type 2 diabetes.[21 22]

In summary, this study disclosed a significant genetic-environmental interaction, with very large ORs, associated with the development of OSCC. Thus, convincing young people to smoke and drink less is likely to reduce the incidence of OSCC. SNP genotyping demonstrated that the *ADH1B* and/or *ALDH2* risk alleles had an interaction with smoking and, especially, alcohol consumption. Analysis of *ADH1B* and *ALDH2* variants would be valuable for secondary prevention of OSCC in high-risk patients who smoke and drink alcohol. Our findings, if replicated in other groups, could demonstrate new pathophysiological pathways for the development of OSCC.

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Competing interests None.

Ethics approval This study was conducted with the approval of the Kyusyu University, Juntendo University, National Cancer Institute, Saitama Cancer Center, Kurume University, and Kurume University, Japan.

Contributors KY, SS, and H Inoue equally contributed to this study. FT, KY, MT, HK, HF, YT, SN, ST, KM, KI, ST, NH, H Ishii, H Inoue and MM jointly designed the study and organised the recruitment of participants. Y Kajiyama, H Igaki, KF, TT, Y Kawashima, and TS organised the recruitment of participants and biological samples. KY conducted the SNP study and the statistical analysis and drafted the manuscript. SS organised the environmental study and the statistical analysis and drafted the manuscript. FT had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Editor's quiz: GI snapshot

Pizza, beer, amylase, lipase and the acute abdomen

CLINICAL PRESENTATION

A previously healthy 16-year-old male student was admitted with acute abdominal pain after eating two large pizzas and drinking five pints (approximately 2.8 l) of beer (alcohol content 4.5%). Initial assessment revealed epigastric tenderness with elevated serum amylase (380 IU/l, normal 30–110 IU/l) and lipase (4398 IU/l, normal 23–300 IU/l) concentrations. There was no free gas on the chest radiograph. The patient developed increasing abdominal pain, tenderness, tachycardia and a lactic acidosis (pH 7.20, lactate 2.91 mmol/l) within 6 h. Contrast-enhanced abdominal CT (figure 1) was done 8 h after admission.

Figure 1 Contrast-enhanced CT scan of the abdomen.

QUESTION

What are the findings on CT?

See page 1560 for answer

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Patient consent Obtained from parent.

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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Abnormal Expression of *PFDN4* in Colorectal Cancer: A Novel Marker for Prognosis

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ABSTRACT

Background. Prefoldin 4 (*PFDN4*) is a transcriptional factor that regulates the cell cycle. *PFDN4* is upregulated in breast tumor and breast cancer cell lines, but its significance in colorectal cancer (CRC) is not fully understood.

Methods. The present study assessed 129 patients who underwent surgery for CRC and assessed three cell lines derived from human CRC. The correlation of gene expression with clinical parameters in patients was assessed by knockdown experiments with these cell lines.

Results. Patients with high *PFDN4* expression had a statistically relatively better prognosis, and those with low *PFDN4* expression showed poorer overall survival than those with high expression. The assessment of *PFDN4* knockdown in the three cell lines demonstrated that the siRNA inhibition resulted in a statistically significant increase in cell growth and invasiveness.

Conclusions. The data strongly suggest that *PFDN4* expression is a prognostic factor in CRC.

Cancer is one of the most prominent illnesses in public health.[1,2] In particular, the incidence of colorectal cancer (CRC) has increased greatly in recent years in concert with a changing lifestyle.[3] The major cause of death from CRC is metastases.[4] Although treatments have recently improved, they fail in approximately one-third of patients, who need an alternative strategy.[2] Predictive markers would be useful for the treatment of CRC patients.

Tumor-promoting oncogenes and tumor suppressors control cell proliferation through cell-cycle regulation, which has been demonstrated in other tumors.[1,5,6] Further identification of the genes responsible for the development and progression of CRC, as well as the understanding of their clinical significances, would improve the diagnosis and treatment of the disease. Characterization of key molecules is particularly promising for the development of novel approaches for the treatment of CRC.

The human *PFDN4* gene, prefoldin 4, is a subunit of the heterohexameric chaperone protein and belongs to the prefoldin family. Previous reports have shown that *PFDN4* is upregulated in breast cancer cell lines and tumor tissues, but its significance in CRC is not fully understood.[7,8]

We investigated the importance of the *PFDN4* gene by analyzing 129 consecutive cases of CRC as well as three CRC cell lines. We propose that*PFDN4* expression is important for the evaluation of prognosis, and we suggest it as a candidate novel marker for CRC prognosis.

MATERIALS AND METHODS

Clinical Tissue Samples

One hundred twenty-four patients (79 men, 50 women) with CRC were registered and underwent curative surgery including distant metastasis at the Medical Institute of Bioregulation at Kyusyu University from 1993 to 2002. Primary CRC specimens and adjacent normal colorectal

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mucosa were obtained from patients after written informed consent had been obtained, in accordance with institutional ethical guidelines.

The surgical specimens were fixed in formalin, processed through graded ethanol, and embedded in paraffin. The sections were stained with hematoxylin and eosin and with Elastica van Gieson stain, and the degree of histological differentiation, lymphatic invasion, and venous invasion were examined. Pieces of all specimens were frozen in liquid nitrogen immediately after resection and kept at −80°C until RNA extraction. None of the patients received chemotherapy or radiotherapy before surgery. After surgery, the patients were followed up with a blood examination, including the tumor markers serum carcinoembryonic antigen and cancer antigen (CA19-9), and imaging modalities such as abdominal ultrasonography, computed tomography, and chest X-ray every 3–6 months. Postoperatively, stage III and IV patients received 5-fluorouracil-based chemotherapy, whereas stage I and II patients principally received no chemotherapy. All these therapies were performed according to the Japanese guidelines.[9] Clinicopathological factors were assessed according to the tumor node metastasis classification of the International Union Against Cancer.[10]

Cell Lines and Culture

Three cell lines derived from human CRC (DLD-1, HCT116, and LoVo) were obtained.[11–13] They were maintained in Dulbecco minimal essential medium (DMEM) containing 10% fetal bovine serum and antibiotics at 37°C in a 5% humidified CO_2 atmosphere. For siRNA inhibition, double-stranded RNA duplexes targeting human *PFDN4,* (5′-AAUCAUCAUCUGCAAGCAUGAUGUC-3′/5′-GACAUCAUGCUUGCAGAUGAUGAUU-3′, 5′-GUUUCUUCUUGAUGAAUGGCUAAUGA-3′/5′-UCAUUAGCCAUUCUCAAGAAGAAAC-3′, and 5′-AGCUUCAAGGUUUAUGUUGCUCCCG-3′/5′-CGGGAGCAACAUAAACCUUGAAGCU-3′), as well as negative control siRNA, were purchased in a Stealth RNAi kit (Invitrogen, Carlsbad, CA). CRC cell lines were transfected with siRNA at a concentration of 20 μmol/L with lipofectamine RNAiMAX (Invitrogen), incubated in glucose-free Opti MEM (Invitrogen), treated in accordance with the manufacturer's protocols, and analyzed by proliferation and invasion assay after 48 h. All siRNA duplexes were used together in a triple transfection. The growth rate of the cultures was measured by counting cells with a CellTac kit (Nihon Koden, Tokyo, Japan). Each cell line with siRNA was compared to the wild type and a negative control. Values were expressed as standard error of mean at least three independent experiments.

RNA Preparation and Quantitative Real-Time Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)

Total RNA was prepared by a modified acid guanidium-phenol-chloroform procedure with DNase.[14] Reverse transcription was performed with 2.5 μg of total RNA as previously described, and a 127-bp *PFDN4* fragment was amplified.[15] Two human *PFDN4* oligonucleotide primers for the PCR reaction were designed as follows: 5′-CGGTAGTCCAGTCCCAAGATG-3′(forward), 5′-TCAGCTCTGTGATTCTACTTGTATTCC-3′ (reverse). The forward primer is located in exon 1, and the reverse primer is located in exon 2. A PCR assay was performed with primers specific to the glyceraladehyde-3-phosphate dehydrogenase (*GAPDH*) gene. The *GAPDH* primers 5′-TTGGTATCGTGGAAGGACTCA-3′ (forward) and 5′-TGTCATCATATTGGCAGGTT-3′ (reverse) produced a 270-bp amplicon. cDNA from human reference total RNA (Clontech, Palo Alto, CA) was used for positive controls. Real-time monitoring of PCR was performed with the LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Tokyo, Japan) for cDNA amplification of *PFDN4* and *GAPDH.* The amplification protocol consisted of 35 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 10 s, and elongation at 72°C for 10 s. The products were then subjected to a temperature gradient from 55°C to 95°C at 0.1°C per second, with continuous fluorescence monitoring to produce product melting curves. The expression ratio of mRNA copies in tumor and normal tissues was calculated and normalized against *GAPDH* mRNA expression.

Invasion Assays

Cell invasion were assessed with CytoSelect Cell Invasion Assay according to the protocol of the manufacture (Cell Biolabs, San Diego, CA) after 48 h of transfection. A total of 1.0×10^5 cells in DMEM were placed on each 8.0-μm pore size membrane insert in 96-well plates. DMEM with 10% fetal bovine serum was placed in the bottom wells. After 24 h, cells that did not invade were removed from the top side of the membrane chamber, and we completely dislodged the cells from the underside of the membrane by tilting the membrane chamber in the cell detachment solution (Cell Biolabs). Lysis Buffer/CyQuannt GR dye solution (Cell Biolabs) were added to each well; the fluorescence of the mixture was read with a fluorescence plate reader at 480 nm/520 nm. The values were expressed as a ratio with wild type (every parental cell).

Immunohistochemistry

Surgical specimens of formalin-fixed, paraffin-embedded tissues from 18 cases of CRC were used for *PFDN4*

immunohistochemistry. After deparaffinization and blocking, the antigen-antibody was incubated overnight at 4°C. Envision reagents (Dako Cytomation, Glostrup, Denmark) were applied to detect the signal from the antigen-antibody reaction. All sections were counterstained with hematoxylin. The primary anti-*PFDN4* rabbit polyclonal antibody (HPA024055; Sigma-Aldrich, St. Louis, MO) was used at a dilution of 1:100. All sections were independently examined for protein expression, assessed by comparison of staining between normal and cancer regions under microscopic examination in each specimen, and scored as positive when cancer regions were stained higher than normal regions.

Statistical Analysis

The relationship between *PFDN4* expression and clinicopathological factors was analyzed with the χ^2 test. Kaplan–Meier survival curves were plotted and compared with the generalized log rank test. Univariate and multivariate analyses to identify prognostic factors were performed by a Cox proportional hazard regression model. The values in vitro assays were analyzed with Wilcoxon rank test. All tests were analyzed with JMP software (SAS Institute, Cary, NC). A P value of <0.05 was considered statistically significant.

RESULTS

Expression of PFDN4 in Clinical Tissue Specimens and Clinicopathological Characteristics

We performed quantitative real-time RT-PCR analysis with primary CRC regions (Suppl. Fig. 1). *PFDN4* expression was calculated by *PFDN4/GAPDH* expression. For the clinicopathological evaluation, experimental samples were divided into two groups according to expression status. Patients with values more than the median *PFDN4* expression value (median, 0.344) were assigned to the high-expression group, and the others were assigned to the low-expression group. Clinicopathological factors related to *PFDN4* expression status of the 129 patients are summarized in Table 1. The data indicated that *PFDN4* expression was correlated with metastasis (M0/M1, $P = 0.037$). Other factors were not statistically significantly correlated with *PFDN4* expression.

TABLE 1 Clinicopathological factors and *PFDN4* mRNA expression in 129 colorectal cancers

Factor	High expression (n = 64), n (%)	Low expression (n = 65), n (%)	*P*
Age (y)			
≥68	30 (46.9)	35 (53.8)	0.428
<68	34 (53.1)	30 (46.2)	
Sex			
Male	39 (60.9)	40 (61.5)	0.944
Female	25 (39.1)	25 (38.5)	
Grade of differentiation			
Well/moderate	58 (90.6)	61 (93.8)	0.494
Other	6 (9.4)	4 (6.2)	
Tumor size			
<30 mm	12 (18.8)	16 (24.6)	0.419
≥30 mm	52 (81.2)	49 (75.4)	
Tumor invasion			
Tis	1 (1.5)	5 (7.7)	0.112
T1	6 (9.4)	5 (7.7)	
T2	6 (9.4)	12 (18.4)	
T3	40 (62.5)	28 (43.1)	
T4	11 (17.2)	15 (23.1)	
Lymph node metastasis			
N0	38 (59.4)	32 (50.0)	0.286
N1–2	26 (40.6)	32 (50.0)	
Lymphatic invasion			
Absent	38 (59.4)	43 (66.1)	0.425
Present	26 (40.6)	22 (33.9)	
Venous invasion			
Absent	52 (81.2)	54 (83.1)	0.786
Present	12 (18.8)	11(16.9)	
Metastasis			
M0	51 (79.7)	41 (63.1)	0.037*
M1	13 (20.3)	24 (36.9)	
UICC stage			
0	1 (1.6)	5 (7.7)	0.059*
I	10 (15.6)	12 (18.5)	
II	22 (34.4)	14 (21.5)	
III	18 (28.1)	11 (16.9)	
IV	13 (20.3)	23 (35.4)	

Well well-differentiated adenocarcinoma, *moderate* moderately differentiated adenocarcinoma, *other* poorly differentiated adenocarcinoma and mucinous carcinoma, *UICC* International Union Against Cancer

*Statistically significant $P < 0.05$, χ^2 test

Relationship Between PFDN4 Expression and Prognosis

The data showed that the postoperative overall survival rate was significantly higher in patients with increased *PFDN4* expression ($P = 0.036$) than in those that did not have increased *PFDN4* expression. The median follow-up was 3.90 years (Fig. 1). Table 2 shows the results of the univariate and multivariate analyses for factors related to postoperative overall survival. In our study, *PFDN4* expression was correlated with distant metastasis (Table 1), and we deselected the factor of distant

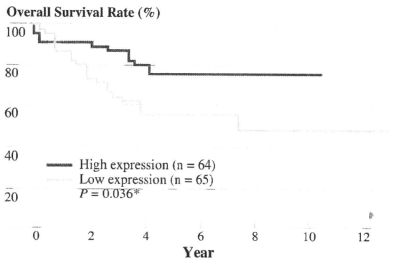

FIG. 1 Overall survival rates of patients with colorectal cancer (CRC) based on *PFDN4* mRNA expression status. The overall survival rate was higher in the *PFDN4* high-expression group than the low-expression group (* $P = 0.036$)

metastasis in univariate and multivariate analysis.[16,17] The univariate analysis showed that age ($P = 0.044$), grade of differentiation ($P = 0.011$), tumor size ($P = 0.006$), tumor invasion ($P < 0.001$), lymph node metastasis ($P < 0.001$), lymphatic invasion ($P < 0.001$), venous invasion ($P < 0.001$), and *PFDN4* expression ($P = 0.035$) were significantly correlated with overall survival. The multivariate regression analysis indicated that inclusion in the *PFDN4* high-expression group (relative risk, 2.68; 95% confidence interval, 1.37–5.49; $P = 0.003$) was an independent predictor of overall survival, as was venous invasion (relative risk, 2.10; 95% confidence interval, 1.01–4.21; $P = 0.045$). Adjuvant chemotherapy was not statistically correlated with overall survival (Suppl. Fig. 2). Univariate and multivariate analyses performed separately for patients with or without adjuvant chemotherapy were assessed (represented in Suppl. Tables 1 and 2). In the analysis of the group with adjuvant chemotherapy, univariate analysis showed *PFDN4* expression ($P = 0.034$) was significantly correlated with overall survival as tumor size ($P < 0.001$), tumor invasion ($P < 0.001$), lymph node metastasis ($P = 0.011$), and venous invasion ($P = 0.042$).

In Vitro Assessment of PFDN4 Expression Knockdown

Three CRC cell lines were chosen for the proliferation and invasion study. In these cell lines, *PFDN4* expression correlated comparatively in clinical samples. The median (range) values of *PFDN4* expression (normalized against *GAPDH* mRNA expression) in these cell lines (DLD-1, HCT116, LoVo) were 0.350 (0.258–0.402), 0.774 (0.589–0.842), and 0.175 (0.128–0.203), respectively. A reduction in *PFDN4* by siRNA was confirmed by quantitative real-time RT-PCR. The proliferation study was confirmed by seeding the cells (1.0×10^5) in six-well dishes and culturing them for 48 h to determine proliferation. The results showed significant differences in HCT116 and LoVo cell numbers between negative control and *PFDN4* siRNA ($n = 3$, $P < 0.05$, Fig. 2a–c). In the invasion study, the results showed significant differences in DLD-1 and LoVo between negative control and *PFDN4* siRNA ($n = 5$, $P < 0.05$, Fig. 2d–f).

Expression of Pfdn4 Protein

The representative immunohistochemical staining pattern for Pfdn4 was shown in tissue from a CRC patient (Suppl. Fig. 3). Pfdn4 protein staining was observed in cytoplasm in epithelial cells; the expression of CRC was compared with noncancerous epithelial cells, whereas the

TABLE 2 Univariate and multivariate analyses for overall survival (Cox proportional hazard regression model)

Factor	Univariate analysis			Multivariate analysis		
	RR	95% CI	*P*	RR	95% CI	*P*
Age (y) (<68/≥68)	1.94	1.01–3.85	0.044*	1.78	0.88–3.77	0.108
Sex (M/F)	1.54	0.79–3.18	0.200			
Grade of differentiation (poor-other/well-moderate)	1.92	1.17–2.89	0.011*	1.68	0.97–2.71	0.058
Tumor size (≥30 mm/<30 mm)	1.97	1.18–4.02	0.006*	1.32	0.75–2.77	0.368
Tumor invasion (T3–4/Tis-2)	4.26	1.98–17.95	<0.001*	2.47	0.99–10.94	0.051
Lymph node metastasis (N1–2/N0)	4.19	2.09–9.08	<0.001*	1.97	0.93–4.49	0.077
Lymphatic invasion (present/absent)	3.49	1.83–6.81	<0.001*	2.00	0.98–4.20	0.054
Venous invasion (present/absent)	3.88	1.94–7.46	<0.001*	2.10	1.01–4.21	0.045*
Adjuvant chemotherapy (present/absent)	1.44	0.76–2.83	0.263			
PFDN4 mRNA expression (median >/≥ median)	2.02	1.04–4.07	0.035*	2.71	1.39–5.52	0.003*

RR relative risk, *95% CI* 95% confidence interval, *well* well-differentiated adenocarcinoma, *moderate* moderately differentiated adenocarcinoma, *poor* poorly differentiated adenocarcinoma, *other* poorly differentiated adenocarcinoma and mucinous carcinoma

*Statistically significant $P < 0.05$, Cox proportional hazard regression model

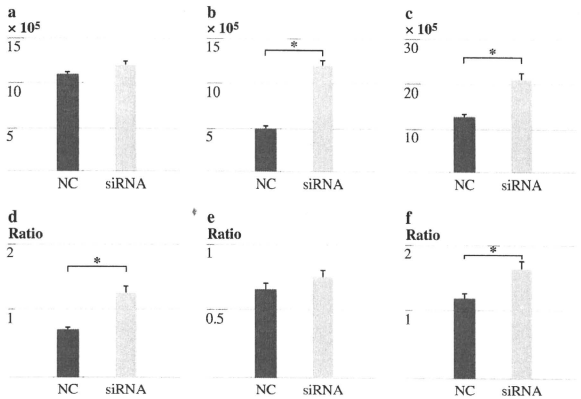

FIG. 2 In vitro assays with siRNA inhibition in the three colorectal cancer (CRC) cell lines. A proliferation assay was performed in 3 CRC cell lines (**a** DLD-1, **b** HCT116, **c** LoVo). There were significant differences between NC and *PFDN4* siRNA in HCT116 and LoVo ($n = 3$, $P = 0.049$ and $P = 0.048$, respectively). An invasion assay was performed in them (**d** DLD-1, **e** HCT116, **f** LoVo). There were significant differences between NC and *PFDN4* siRNA in DLD-1 and LoVo ($n = 5$, $P = 0.009$ and $P = 0.016$, respectively). Invasion assays showed differences in the ratio with wild type (every parental cell). Values are expressed as mean and standard error of the mean. *NC* negative control. * $P < 0.05$

expression was appreciably weak or hardly detectable in stromal cells. Examination of 18 cases, which were selected randomly, indicated that 5 cases exhibited a higher expression level of Pfdn4 protein in cancer regions compared to normal regions (scored as positive), whereas the remaining 13 cases showed no difference between normal and cancer regions (scored as negative). To compare data, *PFDN4* mRNA expression was assessed by real-time RT-PCR in cancer and normal regions in each case. In 5 cases exhibited a higher expression level of Pfdn4 protein, 3 cases showed higher mRNA expression, and it was lower level in 2 of 13 immunohistochemistry-negative cases than normal regions (not statistically significant; χ^2 test).

DISCUSSION

The *PFDN4* gene, also known as *PFD4* or *C1*, is a transcriptional factor regulating the cell cycle.[7,8,18,19] Previous reports indicate that the chaperonin protein coded by *PFDN4* shows cell-cycle-specific expression and is highly expressed in cells in crisis.[18] The *PFDN4* gene is highly expressed in breast cancer cell lines and tumor tissues and has been reported as a candidate tumor-related gene.[7,20] Especially in a previous report about breast cancer, high *PFDN4* expression (relative to *GAPDH* and normalized to human mammary epithelial cells) was shown by RT-PCR analysis (range, 0.1–10 in four breast tumor tissues), and the possibility was indicated that it played a role in cancer behavior, although biological analysis remains to be done.[7]

The present study showed that *PFDN4* expression is an independent prognostic factor for CRC. The siRNA inhibition experiment in the CRC cell lines demonstrated the functional relevance of *PFDN4*. It suggests that the tumor malignancy correlates *PFDN4* expression and it might affect the values of other prognostic factors in multivariate analysis, such as lymph node metastasis, which was statistically significant in univariate analysis (univariate and multivariate analysis resulted in $P < 0.001$ and $P = 0.054$, respectively). *PFDN4* expression correlated with metastasis; however, it was not statistically significant between *PFDN4* expression and venous invasion. These results suggest that *PFDN4* is related to distant metastasis except for tumor invasiveness represented by venous invasion. In our study, we assessed the *PFDN4* expression by immunohistochemistry with anti-*PFDN4* antibody between cancer and normal regions in only 18 samples, but there was no statistically significant difference in the protein and mRNA correlation. To our knowledge, the present study is the first to show *PFDN4* to be a statistically significant predictive marker for CRC prognosis.[21] Furthermore, the results indicated that *PFDN4* plays a role as tumor suppressor, supported by a functional relevance to cell growth and invasiveness. Although downstream targets remain to be understood, our results suggest that *PFDN4*-dependent

pathway might be involved in the suppression of CRC via prefoldin function, which vary their work among types of tissues, such as colon and breast, because chaperone protein coded by *PFDN4* forms a double-barrel assembly with six protruding coiled coils, and it might cause Pfdn4-dependent stabilization of target polypeptides.[8]

It is useful to determine the necessity for intensive follow-up and adjuvant CRC therapy by predicting recurrence and metastases after curative surgical resection.[22–24] In the present study, the clinicopathological analysis revealed that CRC patients with high expression of *PFDN4* showed a better prognosis for overall survival than the low-expression group. The data indicate that *PFDN4* is a presumptive novel predictor of CRC prognosis, especially relating to metastasis. Furthermore, it may be a therapeutic target in CRC from the results of biological experiments in which several cell lines showed lower proliferation and invasiveness than those trasnfected with siRNA. Several adjuvant chemotherapies are helpful in certain disease stages, especially in CRC, and indicate the usefulness of less invasive surgery for CRC.[18–28] For these cases, an informative prognostic marker, which is independent from traditional tumor, node, metastasis system classification and which contributes to diagnoses and treatments, is very important. The present data indicate that *PFDN4* is a good candidate for such a prognostic marker. Although improved preoperative and postoperative treatments, such as chemotherapy and radiotherapy combined with surgery for CRC, have contributed to the reduction of recurrences, eventually, disease in half the cases ultimately metastasizes despite systemic chemotherapy followed by surgery.[29] Adjuvant chemotherapy for CRC is desirable in highly suspicious recurrent cases. In these cases, *PFDN4* analysis may be useful in predicting disease in patients, and in treating patients with a poor prognosis.

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SCRN1 Is a Novel Marker for Prognosis in Colorectal Cancer

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Purpose: Secernin 1 (*SCRN1*) is a member of the secernin family and is reported to be a tumor-associated antigen. Previous reports show that *SCRN1* is upregulated in gastric cancer cell lines and may be a novel immunotherapy target, whereas its significance in colorectal cancer (CRC) is not fully understood.

Materials and Methods: The present study comprised 54 patients who underwent surgery for CRC, as well as two cell lines derived from human CRC. We assessed the correlation of gene expression with clinical parameters in the CRC patients, and knockdown was evaluated in the cell lines.

Results: Thirty of the 54 (55.5%) cases exhibited a higher expression of the *SCRN1* gene in cancerous regions than marginal non-cancerous regions. Patients with high *SCRN1* expression were statistically susceptible to a poor prognosis and showed poorer disease-free survival than those with low expression. *SCRN1* knockdown in the two cell lines demonstrated that the siRNA inhibition resulted in a statistically significant reduction in cell growth.

Conclusion: The present data strongly suggest that *SCRN1* expression is a prognostic factor in CRC patients.
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KEY WORDS: *SCRN1*; prognosis; colorectal cancer

INTRODUCTION

Cancer is one of the most prominent illnesses in the world [1,2]. In particular, the incidence of colorectal cancer (CRC) has increased significantly in recent years in concert with the changing lifestyle [3]. Although the treatment for CRC has improved, it fails in approximately one-third of the patients, who need an alternative strategy according to metastasis [2,4]. Thus, predictive markers would be useful for the treatment of CRC patients.

Tumor-promoting oncogenes and tumor suppressors control cell proliferation through several CRC cellular mechanisms, which are shown in other tumors [1,5,6]. Identifying additional genes responsible for the development and progression of CRC, as well as understanding their clinical significance, would improve diagnosis and treatment of the disease. The characterization of key molecules is particularly promising for the development of novel approaches for the treatment of CRC.

The human secernin 1 (*SCRN1*) gene is a member of the secernin family and is reported to be a tumor-associated antigen in gastric cancer [7,8]. Previous reports have shown that *SCRN1* is upregulated in gastric cancer cell lines and is a novel immunotherapy target, whereas the expression and its significance of prognosis in CRC is not yet understood.

We investigated the importance of the *SCRN1* gene by analyzing it in 54 continuous paired cases of CRC and non-cancerous regions as well as 2 CRC cell lines. We suggest that *SCRN1* expression is important for the evaluation of prognosis and propose that it is a candidate novel marker for CRC prognosis.

MATERIALS AND METHODS

Clinical Tissue Samples

The study included 54 continuous patients who underwent surgery for CRC at Osaka University from 2003 to 2004. Primary CRC specimens and adjacent normal colorectal mucosa were obtained from patients after confirming written, informed consent in accordance with the institutional ethical guidelines of Osaka University. The surgical specimens were fixed in formalin, processed through a graded ethanol series, embedded in paraffin, and the sections were stained with hematoxylin and eosin. All specimens were frozen in liquid nitrogen immediately after resection and kept at −80°C until RNA extractions. After surgery, the patients followed-up with a blood examination including the tumor markers serum carcinoembryonic antigen (CEA) and cancer antigen (CA19-9), and imaging modalities such as abdominal ultrasonography, computed tomography, and chest X-ray were conducted every 3–6 months. Clinico-pathological factors were assessed according to the criteria of the tumor-node-metastasis (TNM) classification of the International Union Against Cancer (UICC) [9].

Cell Lines and Culture

Two cell lines derived from human CRC, DLD-1, and HCT116, were described in the previous reports and adopted in this study [10,11]. They were maintained in Dulbecco's minimal essential medium containing 10% fetal bovine serum and antibiotics at 37°C in a 5% humidified CO_2 atmosphere. For the siRNA inhibition, double-stranded RNA duplexes targeting human *SCRN1*, (5′-UUAUCAUUAUGGCAUAGGUCCUUGG-3′/5′-CCAAGGACCUAUGCCAUAAUGAUAA-3′, 5′-ACAAGGAGACAAUGACAUCUAAGGC-3′/5′-GCCUUAGAUGUCAUUGUCUCCUUGU-3′, and 5′-CAGAUAUGCACUUUGGAAGCUGUGG-3′/5′-CCACAGCUUCCAAAGUGCAU-

Additional Supporting Information may be found in the online version of this article.

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AUCUG-3′), as well as negative control siRNA (NC) were purchased in the Stealth RNAi kit (Invitrogen, Carlsbad, CA). CRC cell lines were transfected with siRNA at a concentration of 20 μmol/L using lipofectamine RNAiMAX (Invitrogen), incubated in glucose-free Opti-MEM (Invitrogen), treated in accordance with manufacturer's protocols, and analyzed by proliferation assay. All siRNA duplexes were used together in a triple transfection. The growth rates of the cultured cells were measured by counting cells using a CellTac kit (Nihon Koden, Tokyo, Japan). siRNA knockdowns were performed in the two CRC cell lines to evaluate proliferation with *SCRN1* suppression. Each cell line and the siRNA were compared to the wild-type and a negative control. Values were expressed as the mean ± standard error of mean (SEM) from five independent experiments.

RNA Preparation and Quantitative Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was prepared using TRIzol reagent and the PureLink RNA Mini Kit (Invitrogen) based on the manufacturer's protocols. Reverse transcription was performed with SuperScriptIII (Invitrogen), and a 139-bp *SCRN1* fragment was amplified. Two human *SCRN1* oligonucleotide primers for the PCR reaction were designed as follows: 5′-GGATGGTCTGGTGGTATTTGG-3′(forward), 5′-CCTTGGAACTTGGTCGATTG-3′ (reverse). The forward primer is located in exon 2 and the reverse primer is located in exon 3. We performed a PCR assay with primers specific to the glyceraladehyde-3-phosphate dehydrogenase (*GAPDH*) gene. The *GAPDH* primers 5′-TTGGTATCGTGGAAGGACTCA-3′ (forward) and 5′-TGTCATCATATTGGCAGGTT-3′ (reverse) produced a 270-bp amplicon. cDNA from human reference total RNA (Clontech, Palo Alto, CA) was used as a source for the positive controls. Real-time monitoring of PCRs was conducted using the LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Tokyo, Japan) to amplify the *SCRN1* and *GAPDH* cDNA. The amplification protocol consisted of 35 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 10 sec, and elongation at 72°C for 10 sec. The products were then subjected to a temperature gradient from 55 to 95°C at 0.1°C sec^{-1}, with continuous fluorescence monitoring to produce product melting curves. The mRNA copy expression ratio in tumor and normal tissues was calculated and normalized against *GAPDH* mRNA expression.

Proliferation Assays

To assess the cell proliferation after 48 hr of siRNA transfection, they were grown for another 48 hr. The cell viability was determined utilizing Cell Counting Kit consisted of WST-8 (Dojin, Tokyo, Japan). Ten microliters of WST-8 was added to the 100 μl medium containing each supplement above, and the absorbance was read at 450 nm using Microplate Reader (Model 680XR, Bio-Rad Laboratories, Hercules, CA). All the experiments were performed at 50–80% cell confluence, and the results were confirmed in five independent experiments. The values were expressed as a ratio/control (every parental cell without transfection).

Statistical Analysis

SCRN1 expression and variables of in vitro analysis were expressed as mean ± SEM and analyzed with the Wilcoxon rank test. The relationship between *SCRN1* expression and clinico-pathological factors was analyzed with the chi-square test. Kaplan–Meier survival curves were plotted and compared with the generalized log rank test. Univariate and multivariate analyses to identify prognostic factors were performed using a Cox proportional hazard regression model. All tests were analyzed with JMP software (SAS Institute, Cary, NC). A P-value <0.05 was considered statistically significant.

RESULTS

Expression of *SCRN1* in Clinical Tissue Specimens and Clinico-Pathological Characteristics

We performed quantitative real-time RT-PCR analysis with paired primary and adjacent non-cancerous CRC regions. RT-PCR on 54 paired clinical samples showed that 30 of these cases (55.5%) exhibited higher levels of *SCRN1* mRNA in tumors than in paired normal tissues (Supplementary Fig. S1).

SCRN1 expression was calculated by dividing the value for *SCRN1*/*GAPDH* expression. For the clinico-pathological evaluation, experimental samples were divided into two groups according to expression status. Patients with values more than the median *SCRN1* expression value (median, 0.190) were assigned to the high-expression group and the others were assigned to the low-expression group. The clinicopathological factors related to *SCRN1* expression status of the 54 patients are summarized in Table I. The data indicated that *SCRN1* expression was not correlated with clinico-pathological factors. In the

TABLE I. Clinicopathological Factors and *SCRN1* mRNA Expression in 54 Colorectal Cancers

Factors	High expression, n = 27 (%)	Low expression, n = 27 (%)	P-value
Age (years)			
<68	11 (40.7%)	13 (48.2%)	0.583
≥68	16 (59.3%)	14 (51.8%)	
Gender			
Male	13 (48.2%)	18 (66.7%)	0.168
Female	14 (51.8%)	9 (33.3%)	
Histological grade			
Wel/Mod	23 (85.2%)	24 (88.9%)	0.684
Others	4 (14.8%)	3 (11.1%)	
Tumor size			
<40 mm	11 (40.7%)	12 (44.4%)	0.783
≥40 mm	16 (59.3%)	15 (55.6%)	
Tumor invasion			
Tis	2 (7.4%)	5 (18.5%)	0.716
T1	2 (7.4%)	2 (7.4%)	
T2	7 (25.9%)	4 (14.8%)	
T3	13 (48.2%)	13 (48.2%)	
T4	3 (11.1%)	3 (11.1%)	
Lymph node metastasis			
N0	19 (70.4%)	19 (70.4%)	1
N1–2	8 (29.6%)	8 (29.6%)	
Lymphatic invasion			
Absent	6 (22.2%)	9 (33.3%)	0.362
Present	21 (77.8%)	18 (66.7%)	
Venous invasion			
Absent	17 (63.0%)	15 (55.6%)	0.579
Present	10 (37.0%)	12 (44.4%)	
Monochronous metastasis			
M0	24 (88.9%)	24 (88.9%)	1
M1	3 (11.1%)	3 (11.1%)	
UICC stage			
0	2 (7.4%)	5 (18.5%)	0.606
I	7 (25.9%)	6 (22.2%)	
IIA	9 (33.3%)	6 (22.2%)	
IIB	1 (3.7%)	2 (7.4%)	
IIIA	1 (3.7%)	0 (0%)	
IIIB	4 (14.8%)	3 (11.1%)	
IIIC	0 (0%)	2 (7.4%)	
IV	3 (11.1%)	3 (11.1%)	

Wel, well-differentiated adenocarcinoma; Mod, moderately differentiated adenocarcinoma; Others, poorly differentiated adenocarcinoma and mucinous carcinoma.

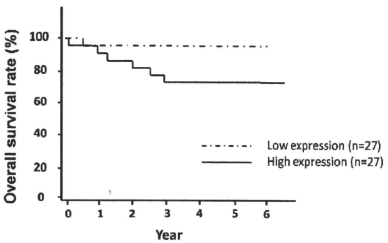

Fig. 1. Overall survival rates of patients with colorectal cancer based on *SCRN1* mRNA expression status. The overall survival rate was lower in the *SCRN1* high-expression group than the low-expression group ($P = 0.049$).

present study, we did not assess the protein expression in relation to the clinic-pathological factors.

Relationship Between *SCRN1* Expression and Prognosis

The data showed that the post-operative overall survival rate was significantly lower in patients with elevated *SCRN1* expression ($P = 0.049$). The median follow-up was 4.21 years (Fig. 1). The disease-free survival rate was lower in patients with elevated *SCRN1* expression ($P = 0.404$, Supplementary Fig. S2). Univariate analysis showed that histological grade ($P = 0.016$), lymph node metastasis ($P < 0.001$), lymphatic invasion (more than maximum repetition, $P = 0.036$), monochronous metastasis ($P < 0.001$), and *SCRN1* expression ($P = 0.039$) were significantly correlated with overall survival (Table II). The multivariate regression analysis revealed that inclusion in the *SCRN1* high-expression group [relative risk (RR), 6.79; 95% confidence interval (CI), 1.02–149.81; $P = 0.046$] was an independent predictor of overall survival.

In Vitro Assessment of *SCRN1* Expression Knockdown

After 48 hr of siRNA transfection two CRC cell lines were evaluated by seeding the cells (1×10^5) in 96-well plates and culturing them for 48 hr to determine proliferation with Cell Counting Kit. The reduction in *SCRN1* by the siRNA was confirmed by quantitative real-time RT-PCR. The results showed significant differences in cell numbers (ratio/parental cells as a reference) between NC and *SCRN1* siRNA ($n = 5$, $P < 0.05$, Fig. 2).

DISCUSSION

The *SCRN1* gene, also known as KIAA0193 or SES1, reportedly contains three secernin genes, termed secernin 1, 2, and 3 [7]. Secernin 1 is located on chromosome 7 (7p14.3-p14.1), and the others are located on chromosomes 17, (17q21.3) and 2 (2p14-q14.3), respectively [7]. Scrn1 is a 50-kDa cytosolic protein involved in the regulation of exocytosis in mast cells, and previous reports show that the *SCRN1* gene is expressed in normal organs such as the brain, prostate, thymus, and intestine [7,8,12]. *SCRN1* is reportedly upregulated in tumor tissues of the stomach and prostate [8,12]. Especially in gastric cancer, a cDNA microarray exhibited *SCRN1* expression in cancer tissues and the cell lines, and *SCRN1* was reported to be a novel immunotherapy target [8].

The present study showed that *SCRN1* expression is an independent prognostic factor for CRC. The siRNA inhibition experiment demonstrated the functional relevance of *SCRN1* in the CRC cell lines. To the best of our knowledge, this is the first study to show *SCRN1* as a statistically significant marker for CRC prognosis [13]. Furthermore, this result was supported by the functional relevance to cell growth with the laboratory-based work.

It is useful to determine the necessity for intensive follow-up and adjuvant therapy for CRC by predicting recurrence and metastases after curative surgical resection [14–16]. In the present study, the clinico-pathological analysis revealed that patients who had CRCs with high *SCRN1* expression had a poorer prognosis for overall survival than the low-expression group. The data indicated that *SCRN1* is presumably a novel predictor of CRC prognosis. Several adjuvant chemotherapies are helpful in certain disease stages, especially in CRC [16–20]. For these cases, an informative prognostic marker, which is independent from the traditional TNM classification, is very important, and contributes to the diagnoses and treatments. Although chemotherapy and radiotherapy combined with surgery for CRC have contributed to the reduction in recurrences, half of cases ultimately metastasize despite systemic chemotherapy followed by surgery [21]. Adjuvant chemotherapy for CRC is necessary for highly suspicious recurrent cases. In these cases, the analysis of *SCRN1* may be useful to predict the prognosis, and *SCRN1* is also expected to be the therapeutic target to treat patients with a poor prognosis.

TABLE II. Univariate and Multivariate Analyses for Overall Survival (Cox Proportional Hazards Regression Model)

	Univariate analysis			Multivariate analysis		
Factors	RR	95% CI	*P*-value	RR	95% CI	*P*-value
Age (years) (<68/≥68)	1.35	0.29–6.89	0.687			
Gender (male/female)	1.32	0.62–2.97	0.462			
Histological grade (por–others/well–mod)	7.9	1.54–36.22	0.016	21.56	—	0.039
Tumor size (≥40/<40)	1.03	0.48–2.34	0.92			
Tumor invasion (T3-4/Tis-2)	4.76	0.81–90.03	0.088			
Lymph node metastasis (N1–2/N0)	19.19	3.23–364.53	<0.001	8.73	0.78–216.68	0.077
Lymphatic invasion (present/absent)	—	—	0.036	—	—	0.192
Venous invasion (present/absent)	4.64	0.99-32.59	0.051			
Monochronous metastasis (M1/M0)	52.5	7.47–1044.07	<0.001	7.11	0.58–175.12	0.123
SCRN1 mRNA expression (>median/≤median)	5.39	1.49–21.77	0.039	6.79	1.02–149.81	0.046

RR, relative risk; CI, confidence interval; Wel, well-differentiated adenocarcinoma; Mod, moderately differentiated adenocarcinoma: Por, poorly differentiated adenocarcinoma; Others, poorly differentiated adenocarcinoma and mucinous carcinoma.
Statistically significant values are underlined.

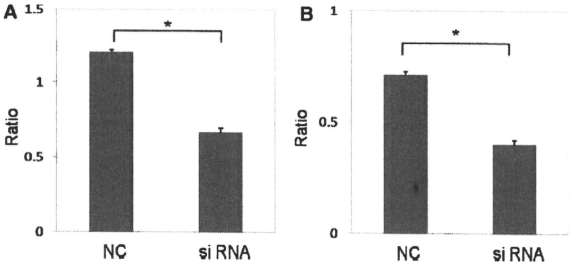

Fig. 2. A proliferation assay was performed in two colorectal cancer cell lines (A, DLD-1; B, HCT116). There were significant differences between NC and *SCRN1* siRNA in DLD-1 and HCT116 ($n = 5$, $^*P = 0.008$, $^*P = 0.009$, respectively). In vitro assays showed differences in the ratio with control (with no treatment) cells. Values are means and SEM. NC, negative control.

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TDGF1 is a novel predictive marker for metachronous metastasis of colorectal cancer

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Abstract. Teratocarcinoma-derived growth factor 1 (*TDGF1*) is a member of the epidermal growth factor-cripto *FRL1* cryptic protein family and is involved in the activation of several different signaling pathways during embryonic development and cellular transformation. Previous reports show that *TDGF1* regulates the activation of several signaling pathways and controls cellular transformation in embryonic status, whereas its significance in colorectal cancer (CRC) is not yet fully understood. The present study comprised 55 patients who underwent surgery for CRC, as well as two cell lines derived from human CRC. The correlation of gene expression with clinical parameters in patients was assessed. The biological significance of *TDGF1* expression was evaluated by knock-down experiments in the cell lines. Seventeen of 55 (30.9%) cases exhibited a higher *TDGF1* expression in cancerous regions than in marginal non-cancerous regions. Patients with high *TDGF1* expression were statistically susceptible to a recurrence of the disease, and showed poorer disease-free survival than those with low expression. The assessment of *TDGF1* knock-down in the 2 cell lines demonstrated that the siRNA inhibition resulted in a statistically significant reduction in cell growth and invasion. In conclusion, the present data strongly suggest the usefulness of *TDGF1* as a predictive marker for metachronous metastasis in CRC patients.

Introduction

Cancer is a prominent malignancy in many developed countries, including the United States and Japan (1,2). The incidence of colorectal cancer (CRC) has increased significantly in recent years in concert with the changing lifestyle (3). The major cause of death from CRC is liver metastases (4). Although treatment has recently improved, it fails in approximately one-third of patients, who require an alternative strategy (2). Thus, useful predictive markers are needed for CRC patients.

Tumor-promoting oncogenes and tumor suppressors control cell proliferation through CRC cell cycle arrest (1,5,6). Identifying additional genes responsible for the development and progression of CRC, as well as understanding their clinical significance would improve diagnosis and treatment of the disease. The characterization of key molecules is particularly promising for the development of novel approaches to treat gastrointestinal tumors.

The human teratocarcinoma-derived growth factor 1 (*TDGF1*) gene is a member of the epidermal growth factor-cripto *FRL1* cryptic gene family and was initially isolated from human teratocarcinoma (7). *TDGF1* is expressed in several types of human tumors and has been detected by immuno-histochemistry in the breast, stomach, colon, pancreas, and lung (8-16). For gastric cancer, the combined expression of *TDGF1* and E-cadherin is reported as a prognostic factor (16).

We investigated the importance of the *TDGF1* gene by analyzing it in 55 consecutive paired cases of CRC and non-cancerous regions as well as in 2 CRC cell lines. We propose that *TDGF1* expression is important for prognostic evaluation and suggest that *TDGF1* could be a novel marker for CRC prognosis.

Materials and methods

Clinical tissue samples. The study comprised 55 consecutive patients who underwent surgery for CRC at Osaka University from 2003 to 2004. Primary CRC specimens and adjacent normal colorectal mucosa were obtained from patients after written, informed consent was confirmed in accordance with the institutional ethics guidelines. The surgical specimens were fixed in formalin, processed through graded ethanol, embedded in paraffin, and sectioned with hematoxylin and eosin. All specimens were frozen immediately in liquid nitrogen after resection and kept at -80˚C until RNA extractions. After surgery, patients were followed up with a blood examination including the tumor markers serum carcinoembryonic antigen (CEA) and cancer antigen (CA19-9), as well as imaging modalities, such as abdominal ultrasonography, computed

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Key words: teratocarcinoma-derived growth factor 1, metastasis, colorectal cancer

tomography, and chest X-ray every 3-6 months. Clinicopathological factors were assessed according to the tumor-node-metastasis (TNM) criteria classification of the International Union Against Cancer (UICC) (17).

Cell lines and culture. Two cell lines derived from human CRC, HCT116 and LoVo, were used in this study (18,19). They were maintained in Dulbecco's minimal essential medium containing 10% fetal bovine serum and antibiotics at 37˚C in a 5% humidified CO_2 atmosphere. For siRNA inhibition, double-stranded RNA duplexes targeting human *TDGF1*, (5'-AAGACUUUAGAAAUGGCCAUGAUCC-3'/5'-GGAUCAUGGCCAUUUCUAAAGUCUU-3', 5'-UUUACUGGUCAUGAAAUUUGCAUGA-3'/5'-UCAUGCAAAUUUCAUGACCAGUAAA-3', and 5'-UGGACGAGCAAAUUCCUGAUGGCCC-3'/5'-GGGCCAUCAGGAAUUUGCUCGUCCA-3'), as well as negative control siRNA (NC) were purchased in the Stealth RNAi kit (Invitrogen, Carlsbad, CA, USA). CRC cell lines were transfected with siRNA at a concentration of 20 μmol/l using lipofectamine RNAiMAX (Invitrogen), incubated in glucose-free Opti-MEM (Invitrogen), treated in accordance with the manufacturer's protocols, and analyzed by proliferation assay. All siRNA duplexes were used together as a triple transfection. The number of cell cultures was measured by counting cells with a CellTac kit (Nihon Koden, Tokyo, Japan). siRNA knockdowns were performed in the two CRC cell lines to evaluate proliferation and invasion under *TDGF1* suppression. Each cell line with siRNA was compared to the wild-type and a negative control. Values were expressed as the mean ± standard error of mean (SEM) from five independent experiments.

RNA preparation and quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was prepared using TRIzol reagent and a PureLink RNA Mini kit (Invitrogen) in accordance with the manufacturer's protocols. RNA was reverse transcribed with SuperScriptIII (Invitrogen), and a 119-bp *TDGF1* fragment was amplified. Two human *TDGF1* oligonucleotide primers for the PCR reaction were designed as follows: 5'-AGATGGCCCGCTTCTCTTAC-3' (forward), 5'-CAGGTATCCCCGAGATGGAC-3' (reverse). The forward primer is located in exon 1 and the reverse primer is located in exon 2. PCR was performed with primers specific to the glyceraladehyde-3-phosphate dehydrogenase (*GAPDH*) gene. The *GAPDH* primers 5'-TTGGTATCGTGGAAGGACTCA-3' (forward) and 5'-TGTCATCATATTGGCAGGTT-3' (reverse) produced a 270-bp amplicon. cDNA from the human reference total RNA (Clontech, Palo Alto, CA, USA) was used as a source of positive controls. Real-time PCR monitoring was performed using the Light Cycler FastStart DNA Master SYBR-Green I kit (Roche Diagnostics, Tokyo, Japan) for *TDGF1* and *GAPDH* cDNA amplification. The amplification protocol consisted of 35 cycles of denaturation at 95˚C for 10 sec, annealing at 60˚C for 10 sec, and elongation at 72˚C for 10 sec. The products were then subjected to a temperature gradient from 55 to 95˚C at 0.1˚C s^{-1} with continuous fluorescence monitoring to produce product melting curves. The expression ratio of mRNA copies in tumor and normal tissues was calculated and normalized against *GAPDH* mRNA expression.

Proliferation and invasion assays. To assess the cell proliferation after 48 h of siRNA transfection, they were grown for another 48 h. The cell viability was determined utilizing Cell Counting kit consisted of WST-8 (Dojin, Tokyo, Japan). WST-8 (10 μl) was added to the 100 μl medium containing each supplement above, and the absorbance was read at 450 nm using Microplate Reader (Model 680XR, Bio-Rad Laboratories, CA). All the experiments were performed at 50-80% cell confluence, and the results were confirmed in five independent experiments. The values were expressed as a ratio/control (every parental cell).

Cell invasion were assessed with CytoSelect Cell Invasion Assay according to the protocol of the manufacture (Cell Biolabs, San Diego, CA) after 48 h of the transfection. Cells ($1.0x10^5$) in DMEM were placed on each 8.0-μm pore size membrane insert in 96-well plates. DMEM with 10% FBS was placed in the bottom wells. After 24 h, cells that did not invade were removed from the top side of the membrane chamber and completely dislodge the cells from the underside of the membrane by tilting the membrane chamber in the Cell Detachment Solution (Cell Biolabs). Lysis Buffer/CyQuant GR dye solution (Cell Biolabs) were added to each well, the fluorescence of the mixture was read with a fluorescence plate reader at 480/520 nm. The values were expressed as a ratio/control (every parental cell).

Statistical analysis. The variable data are expressed as mean ± SEM. The relationship between *TDGF1* expression and clinicopathological factors was analyzed with the χ^2 test. Kaplan-Meier survival curves were plotted and compared with the generalized log-rank test. Univariate and multivariate analyses were performed to identify prognostic factors using a Cox proportional hazard regression model. The Wilcoxon rank test was used to compare differences in *TDGF1* siRNA among the cell lines. All tests were analyzed with JMP software (SAS Institute, Cary, NC, USA). Differences with $p<0.05$ were considered statistically significant.

Results

Expression of TDGF1 in clinical tissue specimens and clinicopathological characteristics. We performed quantitative real-time RT-PCR with paired primary and adjacent non-cancerous CRC regions. RT-PCR on 55 paired clinical samples showed that 17 of these cases (30.9%) exhibited higher levels of *TDGF1* mRNA in tumors than in paired normal tissues. *TDGF1* expression was calculated by dividing *TDGF1*/*GAPDH* expression. For clinicopathological evaluation the experimental samples were divided into 2 groups according to expression status. Patients with values more than the median *TDGF1* expression value (median, 1,960) were assigned to the high-expression group and the others were assigned to the low-expression group. Clinicopathological factors related to the *TDGF1* expression status of the 55 patients are summarized in Table I. The results indicated that *TDGF1* expression was correlated with lymphatic invasion (p=0.041), venous invasion (p=0.010), and metastasis (p=0.052). To examine the correlation with metastasis, which indicated a poor prognosis, the data were divided into monochronous and metachronous metastasis groups, and *TDGF1* expression was evaluated for

Table I. Clinicopathological factors and *TDGF1* mRNA expression in 55 colorectal cancers.

Factors	High expression n=27 (%)	Low expression n=28 (%)	P-value
Age (years)			
≥68	11 (40.7)	16 (57.1)	0.222
<68	16 (59.3)	12 (42.9)	
Gender			
Male	14 (51.8)	17 (60.7)	0.507
Female	13 (48.2)	11 (39.3)	
Histological grade			
Wel/Mod	23 (85.2)	25 (89.3)	0.648
Others	4 (14.8)	3 (10.7)	
Tumor size			
≥50 mm	10 (37.0)	17 (60.7)	0.079
<50 mm	17 (63.0)	11 (39.3)	
Tumor invasion			
Tis	1 (3.7)	6 (21.4)	0.051
T1	0 (0)	4 (14.3)	
T2	6 (22.2)	5 (17.9)	
T3	17 (63.0)	10 (35.7)	
T4	3 (11.1)	3 (10.7)	
Lymph node metastasis			
N0	17 (66.7)	20 (71.4)	0.702
N1-2	9 (33.3)	8 (28.6)	
Lymphatic invasion			
Absent	4 (14.8)	11 (39.3)	0.041
Present	23 (85.2)	17 (60.7)	
Venous invasion			
Absent	11 (40.7)	21 (75.0)	0.010
Present	16 (59.3)	7 (25.0)	
Metastasis			
M0	17 (63.0)	24 (85.7)	0.052
M1	10 (37.0)	4 (14.3)	
UICC stage			
0	1 (3.7)	6 (21.4)	0.133
I	4 (14.8)	8 (28.6)	
IIA	7 (25.9)	5 (17.8)	
IIB	2 (7.4)	1 (3.6)	
IIIA	1 (3.7)	0 (0)	
IIIB	2 (7.4)	2 (7.1)	
IIIC	0 (0)	2 (7.1)	
IV	10 (37.0)	4 (14.3)	

Statistically significant values are underlined. Wel, well differentiated adenocarcinoma; Mod, moderately differentiated adenocarcinoma; Others, poorly differentiated adenocarcinoma and mucinous carcinoma.

Table II. Metastasis and *TDGF1* mRNA expression in the 55 patients.

Factors	High expression n=27 (%)	Low expression n=28 (%)	P-value
Monochronous metastasis			
Absent	24 (88.9)	25 (89.3)	0.052
Present	3 (11.1)	3 (10.7)	
Metachronous metastasis			
Absent	17 (70.8)	24 (96.0)	0.017
Present	7 (29.2)	1 (4.0)	

Underlined values indicate statistical significance.

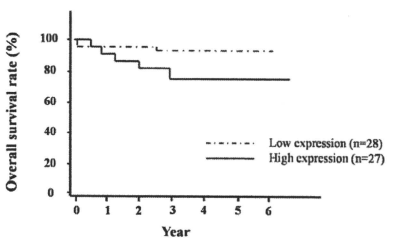

Figure 1. Overall survival rates of colorectal cancer patients based on *TDGF1* mRNA expression status. The overall survival rate was lower in the *TDGF1* high-expression group than the low-expression group (p=0.144).

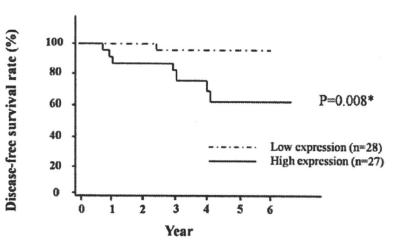

Figure 2. Disease-free survival rates of colorectal cancer patients, exclusive of monochronous metastasis, based on *TDGF1* mRNA expression status. The disease-free survival rate was significantly lower in patients whose samples highly expressed *TDGF1* mRNA than those with lower expression (p=0.008).

each factor (summarized in Table II). The results indicated that *TDGF1* expression was significantly correlated with metachronous metastasis (p=0.017).

Relationship between TDGF1 expression and prognosis. Postoperative overall survival was shorter in patients with elevated *TDGF1* expression (p=0.144) than in those with lower expression. The median follow-up was 4.16 years (Fig. 1). We also evaluated disease-free survival based on the relationship between *TDGF1* expression and metachronous metastasis after

Table III. Univariate and multivariate analyses for disease-free survival in 49 patients with curative surgery (Cox proportional hazards regression model).

Factors	Univariate analysis			Multivariate analysis		
	RR	95% CI	P-value	RR	95% CI	P-value
Age (years)						
≤68/>68	1.84	0.45-9.01	0.391			
Gender						
Male/female	2.17	0.62-18.62	0.192			
Histological grade						
Por-others/well-mod	713.31	-	0.241			
Tumor size (mm)						
≥50/<50	3.34	0.76-22.91	0.110			
Tumor invasion						
T3-4/Tis-2	3.02	0.69-20.70	0.145			
Lymph node metastasis						
N1-2/N0	4.21	0.99-17.85	0.051			
Lymphatic invasion						
Present/absent	-	-	0.014	-	-	0.067
Venous invasion						
Present/absent	2.53	0.59-10.72	0.196			
TDGF1 mRNA expression						
< Median/≥ median	10.42	1.84-195.08	0.005	7.78	1.37-146.02	0.017

Statistically significant values are underlined. RR, relative risk; CI, confidence interval; Wel, well differentiated adenocarcinoma; Mod, moderately differentiated adenocarcinoma; Por, poorly differentiated adenocarcinoma; Others, poorly differentiated adenocarcinoma and mucinous carcinoma.

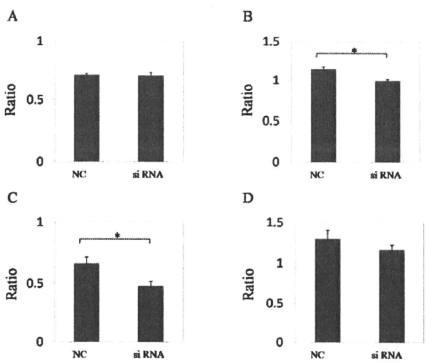

Figure 3. *In vitro* assays with siRNA inhibition in the two colorectal cancer cell lines. A proliferation assay was performed in two colorectal cancer cell lines (A, HCT116; B, LoVo). There were significant differences between NC and *TDGF1* siRNA in LoVo (n=5, *p=0.008). An invasion assay was performed in them (C, HCT116; D, LoVo). There were significant differences between NC and *TDGF1* siRNA in HCT116 (n=5, *p=0.009). *In vitro* assays showed differences in the ratio with control (untreated) cells. Values are means and SEM. NC, negative control.

curative surgery in 49 patients except stage IV at the time of primary operation. The disease-free survival rate was significantly lower in patients with elevated *TDGF1* expression (p=0.008; Fig. 2) than in those with lower expression. Table III shows the univariate and multivariate analyses of factors related to metastatic-free survival in 49 patients. The univariate analysis revealed that *TDGF1* expression (p=0.005) and lymphatic invasion (more than maximum repetition, p=0.014) were significantly correlated with post-operative metastasis. The multivariate regression analysis indicated that inclusion in the *TDGF1* high-expression group (relative risk, 7.78; 95% confidence interval, 1.37-146.02; p=0.017) was an independent predictor of metastatic-free survival.

In vitro assessment of TDGF1 expression knock-down. Two CRC cell lines were chosen for the proliferation and invasion study. A significant reduction in *TDGF1* by siRNA was also confirmed by quantitative real-time RT-PCR. The proliferation study was confirmed by seeding the cells ($1.0x10^5$) in 6-well dishes and culturing them for 48 h to determine proliferation. The results showed significant differences in HCT116 and LoVo cell numbers between NC and *TDGF1* siRNA (n=5, p<0.05, Fig. 3A and B). In the invasion study, the results showed significant differences in DLD-1 and LoVo between NC and *TDGF1* siRNA (n=5, p<0.05, Fig. 3C and D).

Discussion

TDGF1, also known as *CRYPTO*, Crypto-1, or CR-1, is expressed in various cancer tissues of different species (8-16,20-23). Previous *in vitro* and *in vivo* reports show that *TDGF1* regulates signaling pathways and cellular mechanisms as an oncogene (23-26). In mammary tumor, *TDGF1* is associated with molecular mechanisms that contribute to the loss of adherent junctions, referred to as epithelial-mesenchymal transition, which plays an important role in cancer invasiveness and metastasis and might cause a poor prognosis (25-28). The combined expression of *TDGF1* and E-cadherin by immunohistochemistry indicates a poor prognosis in gastric cancer (16).

The present study showed that *TDGF1* expression is an independent predictive factor for metachronous CRC metastasis, and the siRNA inhibition experiment demonstrated the functional relevance of expressed *TDGF1* in the CRC cell lines. To the best of our knowledge, this is the first report to show that *TDGF1* is a predictive marker for CRC metastasis, supported by the functional relevance to cell growth and invasion.

It can be useful to identify the necessity for intensive follow-up and adjuvant therapy by predicting CRC recurrence and metastases after curative surgical resection (29-31). Our clinicopathological analysis revealed that CRC patients with high *TDGF1* expression had a poorer prognosis for disease-free survival than the low-expression group. The results indicated that *TDGF1* is a good predictor for metachronous metastasis, and patients can be followed-up by curative surgical intervention. It is essential to prevent metachronous metastasis during gastrointestinal cancer therapy. Several adjuvant chemotherapies are helpful in particular disease stages, especially in CRC, and indicate the usefulness of a less invasive surgical approach for CRC (31-36). For these cases, a predictive informative marker for tumor recurrence, which is independent from traditional TNM classification and collectively contributes to diagnoses and treatments is very important. While improvement in preoperative and postoperative treatments such as chemotherapy and radiotherapy combined with surgery have contributed to a reduction in the recurrence and metastasis of CRC, half of the cases ultimately metastasize despite systemic chemotherapy followed by surgery (37). Adjuvant chemotherapy for CRC is desirable in highly suspicious metastatic cases. In these cases, an analysis of *TDGF1* may be useful to predict and treat patients with a poor prognosis.

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