embedded in paraffin, and sectioned with hematoxylin and eosin stain and elastica van Gieson stain, and the degree of the histological differentiation, lymphatic invasion, and venous invasion was examined. All specimens were frozen in liquid nitrogen immediately after resection and stored at −80°C until RNA extractions were performed.

None of the patients received chemotherapy or radiotherapy before surgery. After the surgery, the patients were followed up with a blood examination that included the tumor markers carcinoembryonic antigen and cancer antigen, and imaging modalities such as abdominal ultrasound, computed tomography, and chest x-ray every 3 to 6 months. Clinicopathological factors were assessed according to the criteria of the tumor node metastasis classification of the International Union Against Cancer.[10]

Cell Lines and Culture

Seven cell lines derived from human CRC (Caco2, DLD-1, HCT116, HT-29, KM12SM, LoVo, and SW480) were obtained and maintained in Dulbecco modified Eagle medium containing 10% fetal bovine serum and antibiotics at 37°C in a 5% humidified CO_2 atmosphere. For the siRNA knockdown experiment, double-stranded RNA duplexes targeting human *TGM2* (5′-UAGGAUCCCAUCUUCAA ACUGCCCA-3′/5′-UGGGCAGUUUGAAGAUGGGAUC CUA-3′, 5′-AUCCCAUUGUAGCUGACGGUGCGGG-3′/5′-CCCGCACCGUGAGCUACAAUGGGAU-3′, and 5′-UG UAGUUGGUCACGACGCGGGUAGG-3′/5′-CCUACCCG CGUCGUGACCAACUACA-3′) were purchased (Stealth RNAi) from Invitrogen (Carlsbad, CA). Negative control siRNA (NC) was also purchased from Invitrogen. 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) for the time indicated, and analyzed by the proliferation assay. All siRNA duplexes were used together as a triple transfection. siRNA knockdowns were performed in seven CRC cell lines to evaluate proliferation under *TGM2* suppression. Each cell line with siRNA was compared with the negative control. The values are presented as mean ± standard deviation (SD) from independent experiments conducted in triplicate.

RNA Preparation and Quantitative Real-Time Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was prepared by using a modified acid guanidium-phenol-chloroform procedure with DNase.[11] Reverse transcription was performed from 2.5 μg of total RNA as previously described.[12] A 143-bp *TGM2* fragment was amplified. Two human *TGM2* oligonucleotide primers for the polymerase chain reaction (PCR) reaction were designed as follows: 5′-ATAAGTTAGCGCCGCTCTCC-3′ (forward); 5′-CCAGCTCCAGATCACACCTC-3′ (reverse). The forward primer is located in exon 1 and the reverse primer in exon 2. The PCR assay with primers specific to the glyceraladehyde-3-phosphate dehydrogenase (*GAPDH*) gene was performed to evaluate expression. 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) was studied concurrently as a positive control. Real-time monitoring of the PCRs was performed with the LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Tokyo, Japan) for cDNA amplification of *TGM2* and *GAPDH*. The amplification protocol consisted of 35 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 10 seconds, and elongation at 72°C for 10 seconds. 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.

Proliferation Assays

In CRC cell lines transfected with siRNA, 1×10^5 cells were seeded in 12-well dishes and cultured for 96 hours to determine proliferation. The cell growth rate was measured by counting cells with a CellTac kit (Nihon Koden, Tokyo, Japan).

Statistical Analysis

Continuous variable data were expressed as mean ± SD. The relationship between mRNA expression and clinicopathological factors were analyzed by the χ^2 test and Student's *t*-test. Kaplan-Meier survival curves were plotted and compared with the generalized log rank test. Univariate and multivariate analyses to identify prognostic factors for overall survival were performed by the Cox proportional hazard regression model. All tests were analyzed by JMP software (SAS Institute, Cary, NC). *P* values of <.05 were considered statistically significant.

RESULTS

TGM2 mRNA Expression in Clinical Tissue Specimens

Reverse transcriptase–polymerase chain reaction (RT-PCR) of 91 paired clinical samples showed that 65 (71.4%) of the 91 cases exhibited higher levels of *TGM2* mRNA in tumors than paired normal tissues (Fig. 1). The mean *TGM2* mRNA expression value in tumor tissues was

significantly higher than that for corresponding normal tissues ($P = .015$; Student's *t*-test).

TGM2 Expression and Clinicopathological Characteristics

The experimental samples were divided into two groups according to expression status for the clinicopathological evaluation. Patients with tumors that had more than the median *TGM2/GAPDH* expression (median .329) were assigned to the high expression group ($n = 46$); the others were assigned to the low expression group ($n = 45$, Table 1). The number of cases that were based on histological grade was 37, 47, 4, and 3 in the well, moderate, poor, and mucinous adenocarcinoma categories, respectively. *TGM2* expression was correlated with tumor type ($P = .002$), tumor invasion ($P < .001$), lymph node metastasis ($P = .041$), lymphatic invasion ($P = .010$), metastasis ($P = .040$), and International Union Against Cancer stage ($P < .001$).

Relationship Between TGM2 Expression and Prognosis

Postoperative overall survival rate was statistically significantly lower in patients with increased *TGM2* expression (Fig. 2). The median follow-up was 4.12 years. Table 2 provides the univariate and multivariate analyses of factors related to patient prognosis. Univariate analysis showed that histological grade ($P = .040$), tumor type ($P = .003$), tumor size ($P = .004$), tumor invasion

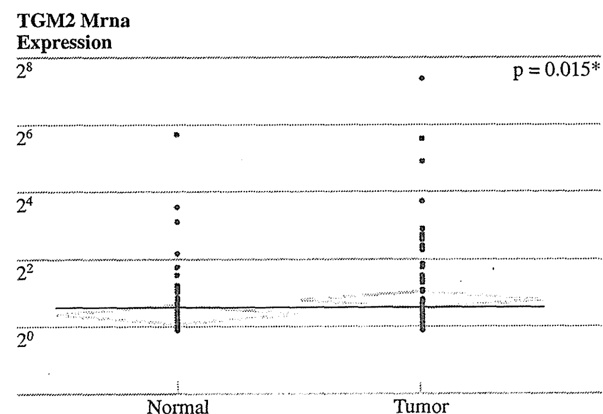

FIG. 1 Transglutaminase 2 (*TGM2*) mRNA expression in clinical tissue specimens. Quantitative real-time reverse transcriptase–polymerase chain reaction of 91 paired clinical samples showed that 65 (71.4%) of the 91 cases exhibited higher levels of *TGM2* mRNA in tumors than in paired normal tissues. The mean *TGM2 m*RNA expression in tumor tissues (normalized by *GAPDH* gene expression) was significantly higher than that of the corresponding normal tissues ($P = .015$; Student's *t*-test)

TABLE 1 Clinicopathological factors and TGM2 mRNA expression in 91 colorectal cancers

Factor	High expression ($n = 46$)	Low expression ($n = 45$)	*P* value
Age (y)			
<68	25 (54.3%)	17 (37.8%)	.112
≥68	21 (45.7%)	28 (62.2%)	
Sex			
Male	31 (67.4%)	31 (68.9%)	.878
Female	15 (32.6%)	14 (31.1%)	
Histological grade			
Wel/Mod	41 (89.1%)	43 (95.6%)	.242
Others	5 (10.9%)	2 (4.4%)	
Tumor type			
0–2	0–2 3 (6.5%)	14 (31.1%)	.002*
3–4	3–4 43 (93.5%)	31 (68.9%)	
Tumor size			
<30 mm	39 (84.8%)	35 (77.8%)	.391
≥30 mm	7 (15.2%)	10 (22.2%)	
Tumor invasion			
Tis	0 (0%)	5 (11.1%)	≤.001*
T1	2 (4.3%)	6 (13.3%)	
T2	3 (6.5%)	12 (26.7%)	
T3	28 (60.9%)	18 (40.0%)	
T4	13 (28.3%)	4 (8.9%)	
Lymph node metastasis			
N0	22 (47.8%)	31 (68.9%)	.041*
N1–2	24 (52.2%)	14 (31.1%)	
Lymphatic invasion			
Absent	24 (52.2%)	35 (77.8%)	.010*
Present	22 (47.8%)	10 (22.2%)	
Venous invasion			
Absent	38 (82.6%)	40 (88.9%)	.392
Present	8 (17.4%)	5 (11.1%)	
Metastasis			
M0	29 (63.0%)	37 (82.2%)	.040*
M1	17 (37.0%)	8 (17.8%)	
UICC stage			
0	0 (0%)	5 (11.1%)	≤.001*
I	5 (10.9%)	12 (26.7%)	
IIA	11 (23.9%)	11 (24.4%)	
IIB	2 (4.3%)	1 (2.2%)	
IIIA	0 (0%)	5 (11.1%)	
IIIB	8 (17.4%)	3 (6.7%)	
IIIC	3 (6.5%)	0 (0%)	
IV	17 (37.0%)	8 (17.8%)	

Wel well differentiated adenocarcinoma, *mod* moderately differentiated adenocarcinoma, *others* poorly differentiated adenocarcinoma and mucinous carcinoma, *UICC* International Union Against Cancer

* Statistically significant

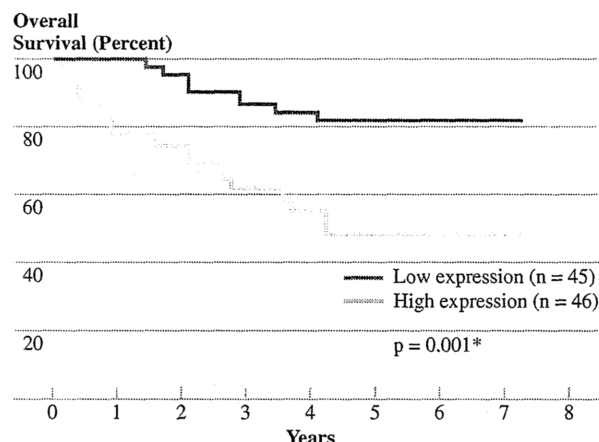

FIG. 2 Overall survival curves of colorectal cancer patients based on *TGM2* mRNA expression status. The postoperative overall survival rate was significantly lower among patients in the high *TGM2* expression group ($P = .001$, log rank test) than the low expression group. The median follow-up was 4.12 years

($P < .001$), lymph node metastasis ($P < .001$), lymphatic invasion ($P = .006$), venous invasion ($P = .001$), and *TGM2* mRNA expression ($P = .003$) were significantly related to overall survival. Multivariate analysis indicated that inclusion in the *TGM2* mRNA high expression group (relative risk, 2.40; 95% confidence interval, 1.03–6.11; $P = .041$) was an independent predictor of postoperative overall survival, as was metastasis (M1/M0, relative risk, 5.86; 95% confidence interval, 2.49–15.12; $P < .001$).

In Vitro Assessment of TGM2 Expression Knockdown

Seven CRC cell lines were used for the proliferation study because *TGM2* expression was higher than the median value of *GAPDH* in the primary CRC specimen by RT-PCR. A reduction in *TGM2* by siRNA was observed by quantitative real-time RT-PCR in all the cell lines examined (negative control [NC] and *TGM2* siRNAs; $P < .05$, Student's *t*-test). A reduction in *TGM2* expression was confirmed in the HT-29, HCT116, KM12SM, and LoVo cell lines (Suppl. Fig. S1). In proliferation assay, there were differences in cell numbers of HT-29 between NC and *TGM2* siRNA ($P < .05$) (Fig. 3). There was no statistically significant difference in the number between the NC and *TGM2* siRNA in the other cell lines.

DISCUSSION

Previous reports showed that *TGM2,* also known as *TG2*, is expressed in breast and pancreatic cancer cells and is associated with drug resistance and metastasis.[4–16] *TGM2* promotes a stable interaction with extracellular matrix protein components in association with some β members of the integrin family of proteins, which induce cell survival signaling pathways.[17] Other reports suggest that *TGM2* regulates activation of NF-κB by forming a ternary complex with NF-κB/IκBα, and inhibition of apoptosis through transamidation and GTP-binding activity.[4,9,18]

Seven distinct transglutaminases have been described.[19–22] *TGM2* is ubiquitously expressed as a single/ polypeptide protein that exhibits Ca^{2+}-dependent protein cross-linking activity.[23]

We assessed *TGM2* gene expression and found that it was a statistically significant independent prognostic factor, similar to the well-known important predictive factor.[24] To our knowledge, the present study is the first report

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

Factor	Univariate analysis			Multivariate analysis		
	RR	95% CI	*P* value	RR	95% CI	*P* value
Age (y), <68/≥68	1.47	0.70–3.11	.298			
Sex, male/female	1.40	0.64–3.38	.401			
Histological grade, por–others/well–mod	3.66	1.06–9.64	.040*	2.52	0.68–7.45	.148
Tumor type, 3–4/0–2	8.27	1.76–147.44	.003*	1.80	0.22–40.49	.615
Tumor size, ≥30 cm/<30 cm	2.82	1.30–11.91	.004*	1.26	0.45–6.02	.697
Tumor invasion, T3–4/Tis-2	7.60	2.27–47.16	≤.001*	1.13	0.36–2.68	.802
Lymph node metastasis, N1–2/N0	5.42	2.43–13.74	≤.001*	2.06	0.83–5.74	.119
Lymphatic invasion, present/absent	2.80	1.34–5.89	.006*	1.32	0.53–3.22	.532
Venous invasion, present/absent	4.20	1.81–9.03	.001*	2.24	0.85–5.80	.099
Metastasis, M1/M0	8.93	4.14–20.84	≤.001*	5.86	2.49–15.12	≤.001*
TGM2 mRNA expression, ≥median/median>	3.08	1.43–7.18	.003*	2.40	1.03–6.11	.041*

RR relative risk, *95% CI* 95% confidence interva, *wel* well-differentiated adenocarcinoma, *mod* moderately differentiated adenocarcinoma, *por* poorly differentiated adenocarcinoma, *others* poorly differentiated adenocarcinoma and mucinous carcinoma

* Statistically significant

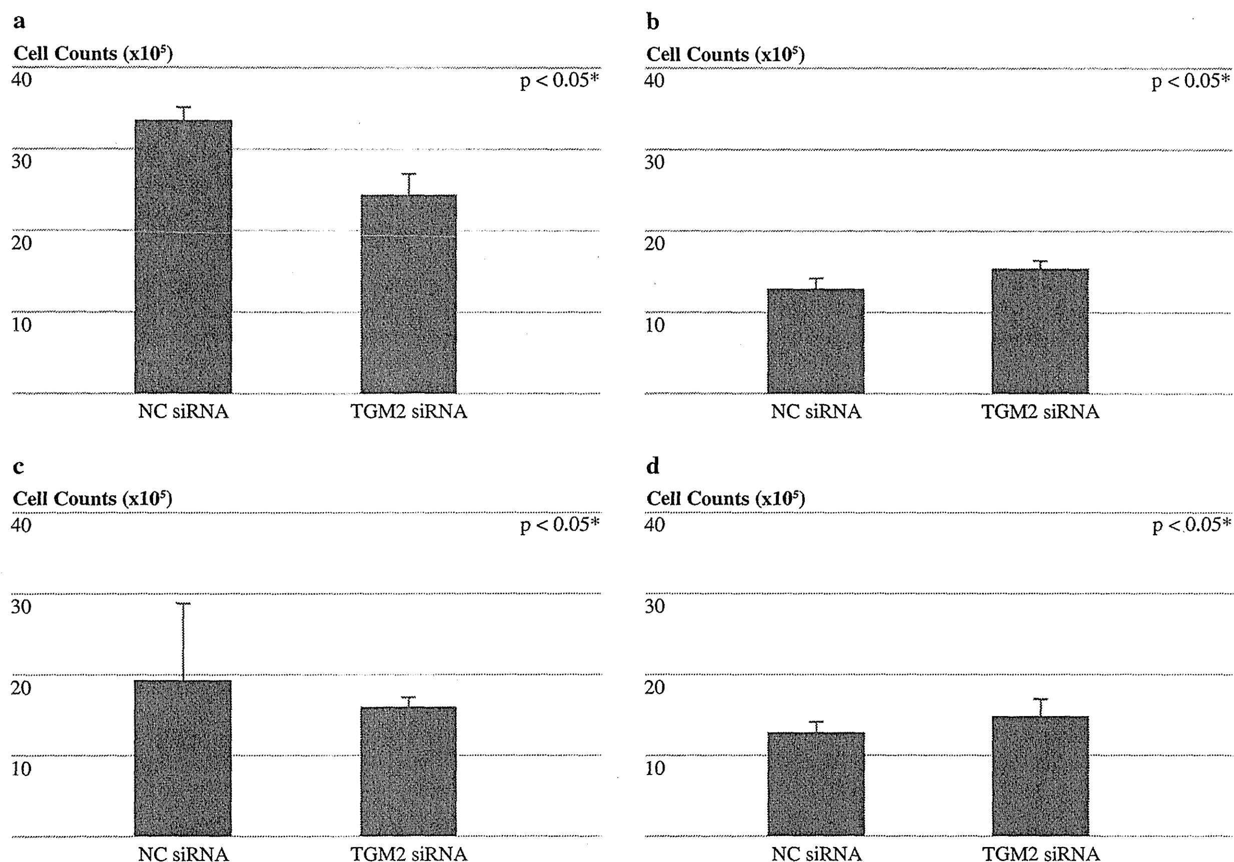

FIG. 3 Proliferation assay and siRNA inhibition in 4 colorectal cancer cell lines. The proliferation assay showed a difference in growth of colorectal cancer cell line HT-29. There were significant differences between NC and *TGM2* siRNA. In the other 3 cell lines, there was no significant difference between NC and *TGM2* siRNA (**a** HT-29; **b** HCT116; **c** KM12SM; **d** LoVo). Values are mean ± SD for three independent experiments. WT, wild type; NC, negative control

showing that *TGM2* is upregulated in CRCs, suggesting that it could be a novel predictive marker for the prognosis of CRCs that may contribute to further clinical cancer diagnosis.

Recently, the necessity of intensive follow-up and adjuvant therapy for CRC has been proposed to predict recurrence and metastasis in curative surgical resected cases.[25–27] In addition, there have been many recent reports on the use of less invasive surgery for CRC such as laparoscopic and endoscopic surgery.[28–31] For these cases, a predictive marker of tumor invasion, lymph node metastasis, and distant metastasis would play a very important role in cancer diagnoses and treatments, especially as a novel marker independent from the traditional tumor, node, metastasis factors. Thus, the *TGM2* expression profile could contribute to the predictive diagnosis of CRCs.

TGM2 plays an important role in antiapoptotic signaling pathways and several cancer cell lines that exhibit high *TGM2* expression levels and have been selected for resistance to chemotherapeutic drugs.[17,32,33] Downregulation of *TGM2* expression by siRNA rendered the cancer cells sensitive to chemotherapeutic drugs.[17]

The present in vitro study showed that *TGM2* expression is associated with tumor growth, and the inhibition of *TGM2* may lead to a reduction in CRC proliferation. *TGM2* is expressed in several cancers.[14–16] Our results suggest a rationale for further study of *TGM2* as a possible novel target for clinical cancer therapy such as anticancer agents and the sensitizer in addition to the novel marker of prognosis and prediction about the susceptibility of anticancer agents.

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Clinical Studies

Abnormal expression of *TRIB3* in colorectal cancer: a novel marker for prognosis

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BACKGROUND: *TRIB3* is a human homologue of *Drosophila tribbles*. Previous studies have shown that *TRIB3* controls the cell growth through ubiquitination-dependent degradation of other proteins, whereas its significance in the prognosis of colorectal cancer (CRC) is not yet fully understood.
MATERIALS: This study comprised 202 patients who underwent surgery for CRC, as well as 22 cell lines derived from human gastrointestinal cancer. The correlation of gene expression with clinical parameters in patients was assessed. The biological significance was evaluated by knockdown experiments in seven colorectal cancer cell lines.
RESULTS: A total of 20 cancer cell lines (90.9%) expressed the *TRIB3* gene. The assessment in surgical specimens indicated that the gene expression was significantly higher in the cancerous region than in the marginal non-cancerous region. Patients with high *TRIB3* expression were statistically susceptible to a recurrence of the disease, and showed poorer overall survival than those with low expression. The assessment of *TRIB3* knockdown in five cell lines showed that small interfering RNA (siRNA) inhibition resulted in a statistically significant reduction in cell growth.
CONCLUSION: These data strongly suggest the usefulness of *TRIB3* as a marker for predicting the prognosis of CRC patients, showing a basis for the development of effective treatments for CRC.
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Keywords: *TRIB3*; prognosis; metastasis; colorectal cancer

In many developed countries, including the United States and Japan, cancer is one of the most prominent illnesses in public welfare and health measures (Jones *et al*, 2007; Jemal *et al*, 2008). The incidence of colorectal cancer (CRC) has increased significantly in recent years, in concert with the changing lifestyle (Kohno *et al*, 2007). The major cause of death in CRC is liver metastases (Yamasaki *et al*, 2007). Although treatment of CRC has improved recently, it fails in approximately one-third of the patients who need an alternative strategy for coping with death (Jones *et al*, 2007). In this matter, useful predictive markers would be desired in the medication of CRC patients.

As shown in other tumours, tumour-promoting oncogenes and tumour suppressors control cell proliferation through cell-cycle arrest of CRC (Aliaga *et al*, 1999; Jemal *et al*, 2008; Yamatodani *et al*, 2009). Further identification of genes responsible for the development and progression of CRC, as well as understanding of their clinical significance, would lead to efficient diagnosis and treatment of the disease. Characterization of key molecules is particularly promising for the development of new approaches for the treatment of gastrointestinal tumours.

Previous studies have shown that chromosomal aberrations occur during carcinogenesis, and relate to patients' prognoses in CRC (Hermsen *et al*, 2002; Leslie *et al*, 2003). Alterations of particular loci at chromosome 20 are reported, indicating the significance of studies on this chromosomal region (Wang *et al*, 2001; Pledgie *et al*, 2005; Yde *et al*, 2007; Goodwin *et al*, 2008; Shor *et al*, 2008). It has been shown that aberrant gains at chromosome 20 are specifically associated with mutations in the tumour suppressor gene, *TP53*, by a survey of 50 cases of CRC, and they are also correlated with the progression of CRC, suggesting that the tumour suppressor pathway is involved in the maintenance of particular chromosomal regions (Wang *et al*, 2001; Leslie *et al*, 2003; Pledgie *et al*, 2005; Yde *et al*, 2007; Goodwin *et al*, 2008; Shor *et al*, 2008).

Although previous studies suggest candidate genes in the regions at chromosome 20, which might have a role in CRC, it is yet to be fully understood in prognostic value (Wu *et al*, 2006; Zheng *et al*, 2008; Antonacopoulou *et al*, 2008). Here we report on *TRIB3* gene in the chromosomal region at 20p13, which is overexpressed in CRC, as a new marker for prognosis and metachronous metastasis. Trib3 is a human homologue of *Drosophila tribbles 3*, which regulates cell growth, differentiation, oogenesis and metabolism by promoting ubiquitination-dependent degradation of other proteins, interacts with several transcriptional factors and is expressed in several tumours (Mata *et al*, 2000; Bowers *et al*,

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2003; Du *et al*, 2003; Koo *et al*, 2004; Boudeau *et al*, 2006; He *et al*, 2006; Koh *et al*, 2006; Matsushima *et al*, 2006; Ord *et al*, 2007; Kato and Du, 2007; Xu *et al*, 2007; Yao and Nyomba, 2008). We studied the *TRIB3* gene in 202 paired cancerous and non-cancerous regions of CRC, as well as 7 colorectal cancer cell lines and 15 other gastrointestinal cancer cell lines. Our data indicate the clinical significance of *TRIB3* in the evaluation of CRC prognosis.

MATERIALS AND METHODS

Cell lines and culture

A total of 22 cell lines derived from human CRC and other gastrointestinal cancer (for CRC: Caco2, DLD-1, LoVo, HCT116, HT-29, KM12SM and SW480; for oesophageal cancer: TE-5, TE-8 and TE-10; for gastric cancer: MKN28 and MKN45; for pancreatic cancer: MIAPaCa-2, PANC-1 and PSN-1; for hepatocellular carcinoma: HuH-7, HepG2, Hep3B, HLE, HLF and PLC; for cholangiocellular carcinoma: HuCCT-1) were maintained in Dulbecco's modied Eagle's medium containing 10% fetal bovine serum and antibiotics at 37°C in a 5% humidified CO_2 atmosphere. For small interfering RNA (siRNA) inhibition, double-stranded RNA duplexes targeting human *TRIB3* (5′-GCGGUUGGAGUUGG AUGACAACUUA-3′ and 5′-GCGUGAUCUCAAGCUGUGUCGCU UU-3′) were purchased as a Validated Stealth RNAi kit (Invitrogen, Carlsbad, CA, USA), as well as negative control siRNA (12935-112, Stealth RNAi Negative Control, Medium GC Duplex, Invitrogen). CRC cell lines were transfected with siRNA at a concentration of 20 μmol ml^{-1} using lipofectamine RNAiMAX (Invitrogen), incubated in glucose-free Opti-MEM (Invitrogen) and analysed using CellTac, a proliferation assay kit (Invitrogen). Values are presented as means ± s.d. from all independent experiments performed in triplicate.

Clinical tissue samples

The study comprised 202 patients who underwent surgery for CRC, including 118 patients at Kyusyu University from 1992 to 2002, and 84 patients at Osaka University from 2002 to 2006. Primary CRC specimens and adjacent normal colorectal mucosa were obtained from patients after written informed consent had been confirmed, in accordance with institutional ethics guidelines. The surgical specimens were fixed in formalin, processed through graded ethanol and embedded in paraffin, and were sectioned with haematoxylin and eosin staining (see the Supplementary Information). For RNA study, all specimens were frozen immediately after resection in liquid nitrogen and were kept at −80°C until RNA extractions. None of the patients received chemotherapy or radiotherapy before surgery. After surgery, patients were followed up with blood examinations including those for tumour markers, such as 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. Clinico-pathological factors were assessed according to the criteria of tumour-node-metastasis (TNM) classification of the International Union Against Cancer (UICC) (Sobin and Fleming, 1997).

RNA preparation and reverse transcriptase PCR (RT-PCR)

Total RNA was prepared using TRIzol reagent (Invitrogen) or with DNase by a modified acid guanidium-phenol-choroform procedure (Mimori *et al*, 1997). Reverse transcription was performed with SuperScriptII (Invitrogen) or with 2.5 μg of total RNA as previously described (Mori *et al*, 1993). A 158-bp *TRIB3* fragment was amplified. Two human *TRIB3* oligonucleotide primers for the PCR reaction were designed as follows: 5′-TGCCCTACAGGC ACTGAGTA-3′ (forward); 5′-GTCCGAGTGAAAAAGGCGTA-3′ (reverse). The forward primer is located in exon 2 and the reverse primer in exon 3. To confirm PCR amplification, 25-35 cycles of PCR reaction were performed using a PCR kit (Takara, Kyoto, Japan) on a Geneamp PCR system 9600 (PE Applied Biosystems, Foster City, CA, USA) with the following parameters: 95°C for 10 s, 60°C for 10 s and 72°C for 60 s. An 8-μl aliquot of each reaction mixture was size-fractionated in a 1.5% agarose gel and visualised using ethidium bromide staining. To confirm RNA quality, a PCR amplification of 270 bp was performed for the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene using the following primers: 5′-TTGGTATCGTGGAAGGACTCA-3′ and 5′-TGTCAT CATATTGGCAGGTT-3′. Human reference complementary DNAs were used as positive controls (Clontech).

Quantitative real-time RT-PCR

For quantitative assessment, quantitative real-time RT-PCR was performed using a kit, LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Tokyo, Japan), for PCR amplification of *TRIB3* and *GAPDH*. The amplification protocol consisted 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 to 95°C with continuous fluorescence monitoring to produce a melting curve of the products. The expression ratios of mRNA copies in tumour and normal tissues were calculated to normalise against *GAPDH* mRNA expression.

Immunohistochemistry

A total of 20 cases of CRC surgical specimens from formalin-fixed, paraffin-embedded tissues were used for Trib3 immunohistochemistry. After deparaffinization and blocking, the antigen-antibody complex was incubated overnight at 4°C. ENVISION reagents (Dako Cytomation, Glostrup, Denmark) were used to detect the signal from the antigen-antibody reaction. All sections were counterstained with haematoxylin. The primary anti-Trib3 rabbit polyclonal antibody (HPA015272; Sigma, St Louis, MO, USA) was used at a dilution of 1:100. All sections were independently examined for protein expression, and assessed by comparison of staining between normal and cancer regions under microscopic examination of ⩾100 fields in each specimen.

Proliferation assay

To determine the proliferative properties, 1.0×10^5 cells were seeded and cultured into each 24-well dish. The cell growth rate was measured by counting cells using a CellTac kit (Nihon Koden, Tokyo, Japan).

Statistical analysis

For continuous variables, data are expressed as mean ± s.d. The relationship between *TRIB3* expression and clinico-pathological factors was analysed using χ^2 and Student's *t*-tests. Kaplan-Meier survival curves were plotted and compared with the generalised log-rank test. Univariate and multivariate analyses for the identification of prognostic factors were performed using a Cox proportional hazard regression model. All tests were analysed using JMP software (SAS Institute, Cary, NC, USA). Differences with *P*-values <0.05 were considered statistically significant.

RESULTS

Expression of TRIB3 in CRC cell lines and clinical tissue specimens

We first studied the expression of *TRIB3* gene, and evaluated it in gastrointestinal cancer cell lines and clinical tissue samples

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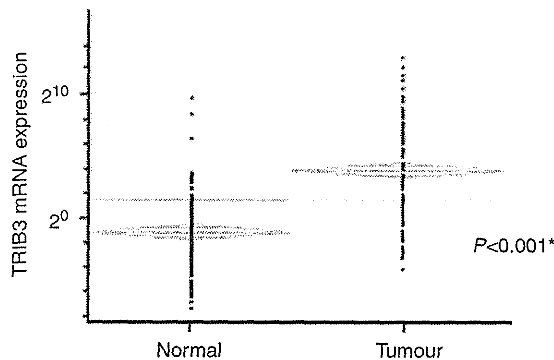

Figure 1 *TRIB3* mRNA expression in clinical tissue specimens. Quantitative real-time RT–PCR on 202 paired clinical samples showed that 181 of 202 (89.6%) samples had higher levels of *TRIB3* mRNA in tumours than in paired normal regions. The mean expression value of *TRIB3* mRNA in tumour regions, 154.62 ± 1021.63 (mean ± s.d.; normalised by *GAPDH* gene expression), was significantly higher than the value, 6.98 ± 4.91, for the corresponding normal regions ($P<0.001$; Student's *t*-test). GAPDH = glyceraldehydes-3-phosphate dehydrogenase; RT–PCR = reverse transcriptase PCR; TRIB3 = tribbles homologue 3.

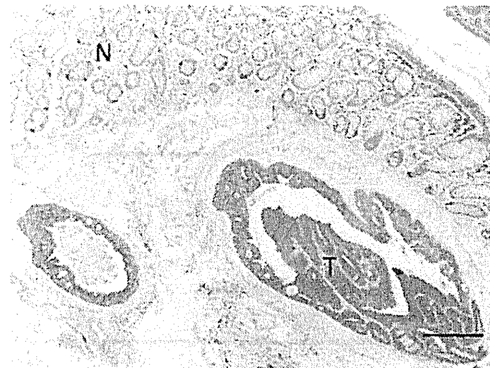

Figure 2 Immunohistochemical staining for Trib3 in tumour and normal specimens. A representative positive stain for Trib3 in tissues from CRC patients. Positive staining is observed in the nucleus and cytoplasm of cancer cells, but not in stromal cells. Trib3 expression was associated with mRNA expression. CRC = colorectal cancer; T = tumour cells; N = normal glandular cells. Bar = 200 μm (original magnification, ×20).

by RT–PCR analysis to confirm that the PCR amplification was specific and produced a single band in agarose gel, stained with ethidium bromide, before performing real-time PCR. The RT–PCR study of *TRIB3* in 22 human gastrointestinal cancer lines indicated 20 cells (90.9%; TE-8, TE-10, MKN45, MIAPaCa-2, PANC-1, PSN-1, HuH-7, HepG2, Hep3B, HLE, HLF, PLC, HuCCT-1, Caco2, DLD-1, LoVo, HCT116, HT-29, KM12SM and SW480) that expressed the *TRIB3* gene with a band in gel (the Supplementary Figure S1A). The RT–PCR analysis of *TRIB3* in primary CRC samples was then performed in paired normal and tumour samples (representative data shown in Supplementary Figure S1B: *TRIB3* expression was higher in cancerous regions than in normal regions). Quantitative real-time RT–PCR on 202 paired cancer and normal samples showed that 181 of 202 (89.6%) samples had higher levels of *TRIB3* mRNA in cancerous regions than in normal regions (Figure 1). The mean expression value of *TRIB3* mRNA in cancerous regions (normalised by *GAPDH* gene expression) was significantly higher than the value in the corresponding normal regions ($P<0.001$; Student's *t*-test).

Expression of Trib3 protein

Figure 2 shows a representative immunohistochemical staining pattern for Trib3 in tissue from a CRC patient. Trib3 protein staining was observed in the nucleus and cytoplasm in epithelial cells; the expression of CRC was compared with non-cancerous epithelial cells, whereas the expression was appreciably weak or hardly detectable in stromal cells. Examination of 20 cases, which were selected randomly, indicated that 16 cases showed a higher expression level of Trib3 protein in cancerous regions compared with normal regions, whereas the remaining four cases showed no difference between normal and cancerous regions. To compare the data, mRNA expression was assessed by gel RT–PCR and real-time RT–PCR. The data show that mRNA expression was high level in all 16 immunohistochemistry-positive tumours, whereas mRNA expression was comparable in normal and cancerous regions of the remaining four tumours, suggesting that the high expression of Trib3 protein is associated with mRNA expression ($P<0.001$; χ^2 test). No variation of staining intensity for Trib3 was observed in each of the specimens. We concluded that both mRNA and the protein coded by this gene are associated and frequently expressed together in CRC.

Table 1 Clinicopahological factors and *TRIB3* mRNA expression in 202 colorectal cancers

Factors	High expression (%)	Low expression (%)	*P*-value
Age (years)			
⩽67	45 (44.5)	51 (50.5)	0.397
67<	56 (55.5)	50 (49.5)	
Gender			
Male	63 (62.4)	52 (51.5)	0.118
Female	38 (37.6)	49 (48.5)	
Histological grade			
Wel-Mod	95 (94.1)	89 (88.1)	0.138
Others	6 (5.9)	12 (11.9)	
Tumour size (mm)			
⩽30	24 (23.8)	22 (21.8)	0.737
30<	77 (76.2)	79 (78.2)	
Tumour invasion			
Tis	5 (5.0)	10 (9.9)	0.418
T1	6 (5.9)	11 (10.9)	
T2	20 (19.8)	19 (18.8)	
T3	49 (48.5)	44 (43.6)	
T4	21 (20.8)	17 (16.8)	
Lymph node metastasis			
N0	60 (59.4)	61 (60.4)	0.885
N1–2	41 (40.6)	40 (36.6)	
Lymphatic invasion			
Absent	49 (48.5)	51 (50.5)	0.778
Present	52 (51.5)	50 (49.5)	
Venous invasion			
Absent	78 (77.2)	74 (73.3)	0.514
Present	23 (22.8)	27 (26.7)	
Metastasis			
M0	68 (67.3)	90 (89.1)	<0.001
M1	33 (32.7)	12 (10.9)	

Wel = well differentiated adenocarcinoma; Mod = moderately differentiated adenocarcinoma; Others = poorly differentiated adenocaricinoma and mucinous carcinoma; TRIB3 tribbles homologue 3. The statistic significance is shown with under line.

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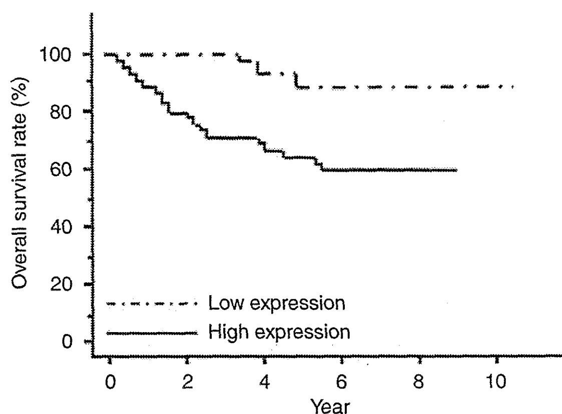

Figure 3 Overall survival rates of patients with CRC on the basis of TRIB3 mRNA expression status. The overall survival rate was significantly lower in the *TRIB3* high-expression group than that in the low-expression group ($P<0.001$). CRC = colorectal cancer; TRIB3 = tribbles homologue 3.

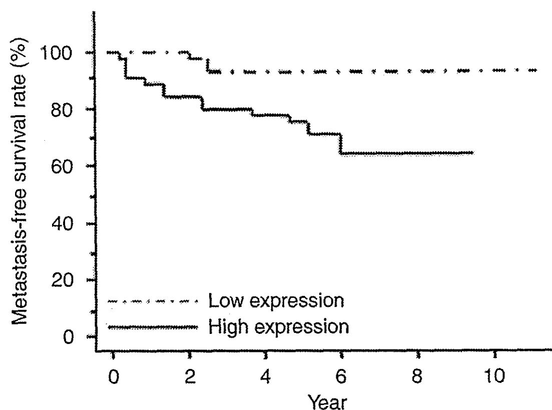

Figure 4 Metachronous metastasis-free over 5 years' survival rates of patients with CRC in stages I II, and III, on the basis of TRIB3 mRNA expression status. The metachronous metastasis-free over 5 years' survival rate was significantly lower in patients with the *TRIB3* high-expression group compared with the low-expression group ($P=0.007$). CRC = colorectal cancer; TRIB3 = tribbles homologue 3.

Table 2 Univariate and multivariate analysis 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) (⩽67/67<)	1.23	0.85–1.80	0.258			
Gender (Male / female)	1.93	0.90–4.47	0.090			
Histological grade (Wel-Mod / others)	1.54	0.36–4.35	0.511			
Tumour size (30</⩽30)	3.70	1.69–15.66	0.001	2.04	0.88–8.79	0.103
Tumour invasion (T3–4 / Tis-2)	11.00	3.28–68.37	<0.001	2.47	0.60–16.82	0.223
Lymph node metastasis (N1–2 / N0)	4.28	2.02–9.63	0.001	1.49	0.65–3.74	0.348
Lymphatic invasion (Present / absent)	2.44	1.14–5.44	0.021	1.43	0.62–3.49	0.396
Venous invasion (Present / absent)	2.17	0.92–4.73	0.071			
Metastasis (M1 / M0)	21.89	9.33–60.11	<0.001	9.34	3.70–27.28	<0.001
TRIB3 mRNA expression (median< / <median)	8.45	2.97–35.48	<0.001	3.78	1.27–16.35	0.014

RR = relative risk; CI = confidence interval; Wel = well differentiated adenocarcinoma; Mod = moderately differentiated adenocarcinoma; Others = poorly differentiated adenocaricinoma and mucinous carcinoma; TRIB3 tribbles homologue 3. The statistic significance is shown with under lines.

Table 3 Univariate and multivariate analysis for metachronous metastasis-free over 5 years survival rate (Cox proportional hazards regression model)

	Univariate analysis			Multivariate analysis		
Factors	**RR**	**95% CI**	***P*-value**	**RR**	**95% CI**	***P*-value**
Age(years) (67</⩽67)	1.33	0.85–2.09	0.202			
Gender (Male / female)	2.44	0.97–6.90	0.055			
Histological grade (Wel-Mod / others)	24.0	4.78–101.61	<0.001	25.9	3.57–215.84	0.001
Tumor size (30< / ⩽30)	3.66	1.66–15.55	<0.001	3.04	1.18–13.62	0.017
Tumor invasion (T3–4 / Tis-2)	4.80	1.61–20.58	0.003	2.77	0.67–15.00	0.160
Lymph node metastasis (N1–2 / N0)	4.01	1.65–10.26	0.002	2.65	0.98–7.59	0.054
Lymphatic invasion (Present / absent)	4.49	1.73–13.83	0.001	0.72	0.20–2.95	0.637
Venous invasion (Present / absent)	3.10	1.21–7.53	0.019	1.68	0.61–4.53	0.301
TRIB3 mRNA expression (median< / ⩽median)	4.33	1.45–18.59	0.006	3.86	1.09–19.00	0.035

RR = relative risk; CI = confidence interval; Wel = well differentiated adenocarcinoma; Mod = moderately differentiated adenocarcinoma; Others = poorly differentiated adenocaricinoma and mucinous carcinoma; TRIB3 tribbles homologue 3. The statistic significance is shown with under lines.

TRIB3 expression and clinico-pathological characteristics

To study the *TRIB3* expression in CRC quantitatively, the data were classified into two experimental groups on the basis of the *TRIB3* expression levels to assess the expression value without any bias. The high-expression group comprised patients who had a level of *TRIB3* expression higher than the median value for *TRIB3/GAPDH* expression in tumour regions compared with normal

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regions ($n = 101$); other patients were assigned to the low-expression group ($n = 101$). Clinico-pathological factors related to *TRIB3* expression status are shown in Table 1. Data indicated that metastasis (M0 / M1) was correlated with *TRIB3* expression ($P < 0.001$). The metastatic sites were the liver (37 cases), lung (10 cases), brain (3 cases) and bone (1 case). Metastatic sites and other factors were not significantly correlated with *TRIB3* expression.

Relationship between TRIB3 expression and prognosis

The study of prognosis revealed that the overall survival rate was significantly lower for patients in the high-expression group ($P < 0.001$; Figure 3). The median follow-up was 2.98 years. Table 2 shows the univariate and multivariate analyses of factors related to patient prognosis. Univariate analysis showed that the post-operative overall survival was significantly correlated with following factors: tumour size ($P = 0.001$), tumour invasion ($P < 0.001$), lymph node metastasis ($P = 0.001$), lymphatic invasion ($P = 0.021$), metastasis ($P < 0.001$) and *TRIB3* expression ($P < 0.001$). Multivariate regression analysis indicated that an inclusion in the *TRIB*3 high-expression group (relative risk (RR) = 3.78; 95% confidence interval (CI) = 1.27 - 16.35; $P = 0.014$) was an independent predictor of overall survival, as was metastasis (M1 / M0) (RR = 9.34; 95% CI = 3.70 - 27.28; $P < 0.001$), indicating a significant link between *TRIB3* expression and patient prognosis.

In 65 of 202 patients, we have followed up over 5 years after the primary operation, the median follow-up was 6.31 years. We then evaluated the metachronous, metastasis-free, over 5years' survival in these patients, indicating that the rate was significantly lower in patients of the high-expression group ($P = 0.007$, Figure 4). Table 3 shows the univariate and multivariate analyses of factors related to patient prognosis. Univariate analysis showed that the post-operative metastasis was significantly correlated with following factors: histological grade ($P < 0.001$), tumour size ($P < 0.001$), tumour invasion ($P = 0.003$), lymph node metastasis ($P = 0.001$), lymphatic invasion ($P = 0.001$), venous invasion ($P = 0.019$) and *TRIB3* mRNA expression ($P = 0.006$). Multivariate regression analysis indicated that inclusion in the *TRIB3* high-expression group (RR = 3.86; 95% CI = 1.09 - 19.00; $P = 0.035$) was an independent predictor of metastasis-free survival, as were histological grade (RR = 25.9; 95% CI = 3.57 - 215.84; $P = 0.001$) and tumour size (RR = 3.04; 95% CI = 1.18 - 13.62; $P = 0.017$).

Effect of TRIB3 inhibition in CRC cell growth

A total of 7 CRC cell lines were subjected to siRNA knockdown. The biological role of *TRIB3 in vitro* was analysed in CRC, in which *TRIB3* expression was knocked down. In the CRC cell lines examined, significant suppression of endogenous *TRIB3* expression by siRNA was confirmed by real-time RT-PCR in five cell lines (DLD-1, LoVo, HCT116, KM12SM and SW480; $P < 0.05$, Student's *t*-test; Supplementary Figure S2). To determine the proliferative properties, cells were seeded and cultured (Figure 5). There were significant differences in numbers between wild-type or negative control and *TRIB3* siRNA ($P < 0.05$) in all five CRC cell lines. There was no significant change in number between negative control and wild type.

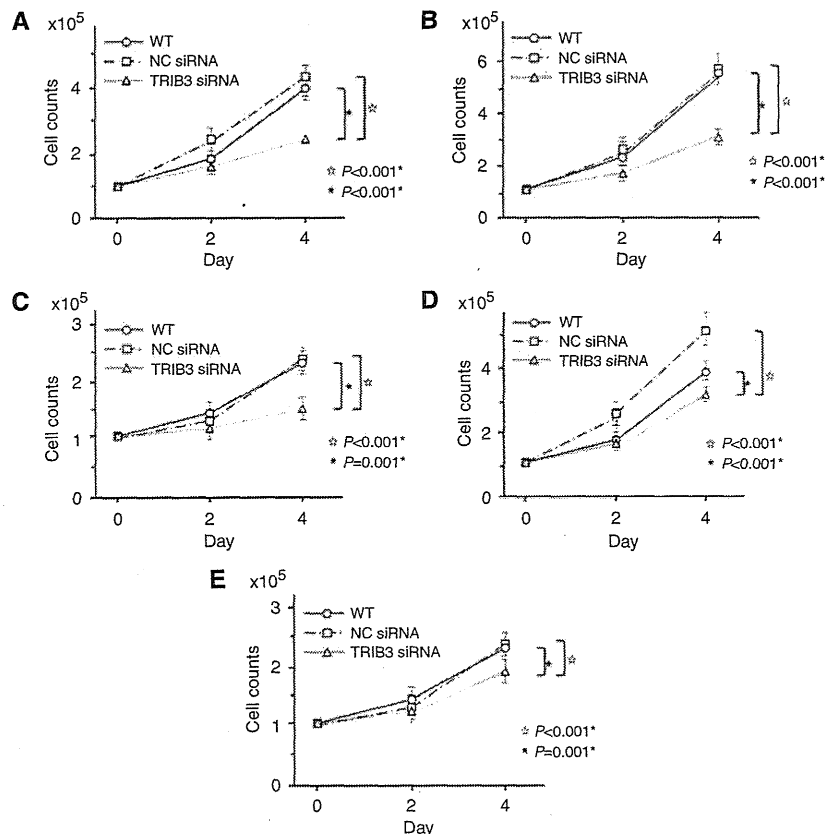

Figure 5 Proliferation assay with siRNA inhibition in five CRC cell lines. Proliferation assay was performed in five CRC cell lines (**A**, DLD-1; **B**, LoVo; **C**, HCT-116; **D**, KM12SM; E, SW480). There were significant differences between WT or NC, and *TRIB3* siRNA. Values are presented means ± s.d. of three independent experiments. CRC = colorectal cancer; NC = negative control; TRIB3 = tribbles homologue 3; WT = wild type.

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DISCUSSION

This study showed that *TRIB3* is expressed at higher levels in CRC than in the corresponding normal regions, and is expressed in gastrointestinal cancer cell lines. The siRNA inhibition experiment showed the functional relevance of expressed *TRIB3* in gastrointestinal cancer cell lines. To the best of our knowledge, this study is the first to show the candidacy of *TRIB3* as a prognostic CRC marker, supported by the functional relevance to cell growth.

Nowadays, it can be useful to determine the necessity of intensive follow-up and adjuvant therapy for CRC by predicting recurrence and metastases in curative surgical resection (Bathe *et al*, 2004; Kornmann *et al*, 2008; Wolpin and Mayer, 2008). In this study, clinico-pathological analysis revealed that TRIB3 is closely related to metastasis, but not to lymphatic metastasis. It may correlate with some mechanism of little concern to invasiveness. Patients with CRCs with high *TRIB3* expression showed a poorer prognosis for disease-free and overall survival than those in the low-expression group. Data indicate that *TRIB3* is an independent prognostic factor, as well as a very important predictor that is already known (Derkinderen *et al*, 1990). *TRIB3* is presumably a good predictor of metachronous metastasis that can be followed by curative surgical intervention. In gastrointestinal cancer therapy, it is essential to prevent metachronous metastasis. Several adjuvant chemotherapies are helpful in certain disease stages, especially in CRC (Bathe *et al*, 2004; Andre *et al*, 2007). Recently, increasing evidence has been accumulated, showing the usefulness of less invasive surgery in the treatment of CRC, such as laparoscopic and endoscopic surgery (Lacy *et al*, 2002; Weeks *et al*, 2002; Clinical Outcomes of Surgical Therapy Study Group, 2004; Jayne *et al*, 2007). For these cases, predictive markers of tumour invasion and metastasis, which are independent of traditional TNM classification and contribute collectively to diagnoses and treatments, are very important. These data indicate the candidacy of *TRIB3*.

Although improving treatments such as pre-operative and post-operative chemotherapy and radiotherapy combined with surgery for CRC have contributed to the reduction of recurrences and metastases, half of the cases eventually metastasise despite systemic chemotherapy followed by surgery (Koshariya *et al*, 2007). Adjuvant chemotherapy for CRC has been desirable in highly suspicious metastatic cases. In these cases, the assessment of *TRIB3* expression may be useful to predict patient prognosis.

In biological assessment, this study showed that *TRIB3* expression was related to tumour growth in several gastrointestinal cancer cell lines. The *in vivo* study showed that siRNA inhibition of *TRIB3* resulted in a reduction in cell growth of seven gastrointestinal cancer cell lines, significantly ($P<0.05$). Although previous reports showed that *TRIB3* is expressed in several cancer cell lines, this study shows that *TRIB3* seems to stimulate proliferation, and may be a new target for the therapy of gastrointestinal cancer (Bowers *et al*, 2003; Xu *et al*, 2007).

Tribs, belong to the pseudokinase family consisting of three mammalian isoforms, Trib1, Trib2 and Trib3, have no detectable kinase catalytic activity because of variations in key amino acids in the ATP-binding domain, but possess substrate-binding domains relating to their function as protein-interacting modules (Seher and Leptin, 2000; Yamatodani *et al*, 2009). Tribs associate with large proteins such as transcriptional factors, and regulate cell growth, differentiation and metabolism (Boudeau *et al*, 2006).

Trib1 interacts with Mapk and modulates Mapkk activity associated with smooth muscle cell proliferation and migration (Kiss-Toth *et al*, 2004; Sung *et al*, 2007). Trib2 has a role in adipogenesis in combination with the degradation of C/EBPbeta (Naiki *et al*, 2007). Trib3 promotes ubiquitination and degradation of proteins involved in cell-cycle regulation and oogenesis through an interaction with activation transcription factor 4, and is involved in the Pten pathway through interaction with Akt (Mata *et al*, 2000; Du *et al*, 2003; He *et al*, 2006; Koh *et al*, 2006; Kato and Du, 2007; Yao and Nyomba, 2008). Trib3 expression is increased in several primary tumours and cancer cell lines and can be controlled by nutrient starvation, which is consistent with these data (Bowers *et al*, 2003; Schwarzer *et al*, 2006; Xu *et al*, 2007). Our report indicates that *TRIB3* is not only a new independent prognostic factor and predictor of metachronous metastasis, but is also a useful target because the inhibition of *TRIB3* may lead to the reduction of CRC through the control of cell growth.

Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)

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Over- and under-expressed microRNAs in human colorectal cancer

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Abstract. MicroRNAs (miRNAs) constitute a class of small (21-23 nucleotides) noncoding RNAs that function as post-transcriptional gene regulators. It is becoming increasingly clear that altered miRNA expression correlates with the pathogenesis of cancers. The purpose of this study was to determine the up-regulated miRNAs in human colorectal cancer. Total RNA was isolated from cancer tissues and corresponding noncancerous tissues from surgically resected colorectal cancers. The expression profiles of miRNAs were determined using a miRNA microarray containing 455 human miRNA probes. The expression status of selected miRNAs in paired clinical samples was then investigated by real-time RT-PCR. Twenty-one miRNAs were identified by miRNA array analysis as overexpressed in colorectal cancer tissues compared to normal epithelial tissues. Among them, the expression of *miR-31*, *miR-183*, *miR-17-5p*, *miR-18a*, *miR-20a* and *miR-92* were confirmed to be significantly higher in cancer tissues than in normal tissues (P<0.05). In contrast, the expression of *miR-143* and *miR-145* in cancer tissues were significantly lower than in normal tissues (P<0.05). The *miR-18a* overexpression group tended to have a poorer clinical prognosis than the low expression group (P=0.07). We identified miRNAs that were overexpressed or under-expressed in colorectal cancers and which may be correlated with colorectal carcinogenesis.

Introduction

MicroRNAs (miRNAs) are evolutionarily-conserved, endogenous, small, noncoding RNA molecules of ~21-23 nucleotides that function as post-transcriptional gene regulators (1-4). Mature miRNAs are integrated into a ribonucleoprotein complex called the RNA-inducing silencing complex (RISC) and associate with 3'-untranslated regions (3'UTRs) of specific target messenger RNAs (mRNAs) to suppress translation and also to occasionally induce mRNA decay (5-9). It is estimated that vertebrate genomes encode up to 1,000 unique miRNAs, each of which is thought to regulate the expression level of a target gene (10). Up to 30% of human genes are thought to be regulated by miRNAs; however, most of the targets remain unknown (11). Recent evidence has shown that miRNAs are involved in regulation of cellular development, differentiation, proliferation and apoptosis (12).

More than 500 miRNAs have been identified in humans and more than half of human miRNAs are located at specific chromosomal regions, including fragile sites, as well as in regions that are frequently amplified, deleted, or rearranged in cancers (13,14). Recent evidence has shown that altered expression of miRNAs is associated with the pathogenesis of various human cancers and has indicated that some miRNAs may function as oncogenes or tumor suppressors (15-20). A number of studies were recently published that focus on the significance of miRNAs in colorectal cancer (21-26). Although assays such as Northern blots and real-time RT-PCR are important in understanding the expression status of individual miRNAs, comprehensive microarray analysis using clinical samples is needed to elucidate the clinical significance of miRNAs in colorectal cancer.

In this study, a microRNA microarray containing 455 human miRNA probes was used to determine expression profiles in colorectal cancer tissue and 21 up-regulated colorectal cancer-related miRNAs were identified. Expression of *miR-31* in cancers was significantly higher than in normal tissues on 69 clinical colorectal cancers by real-time RT-PCR, suggesting that *miR-31* may be one of the potent colorectal cancer-related miRNAs. The *MiR-17-92* cluster may also play an important role in colorectal cancer progression. Furthermore, we demonstrate that *miR-18a* expression could be used as a prognostic factor in predicting survival of colorectal cancer patients.

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Key words: microRNA, human colorectal cancer, microRNA microarray

Materials and methods

Patients and clinical samples. Samples of cancerous tissue and matched noncancerous tissues were obtained from 69 patients with colorectal cancer who underwent surgical resection at Kyushu University Hospital (Beppu, Japan). None of the patients received preoperative treatments, such as radiation and/or chemotherapy. The follow-up periods ranged from 0.1 to 11.3 years with a mean of 3.7 years. Written informed consent was obtained from all patients according to the guidelines approved by the Institutional Research Board and this study was conducted under the supervision of the ethical board of Kyushu University.

The 69 tumor samples and the matched control samples taken from normal tissue located at a distance from the colorectal cancer were frozen in liquid nitrogen immediately after surgical resection and were stored at -90˚C until RNA extraction.

MicroRNA microarray analysis. Total RNAs from tumor and the matched control samples of 4 of 69 cases were analyzed by microRNA microarray. Total RNA was extracted from tissue using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Concentration and purity of the total RNAs were assessed by a spectrophotometer and RNA integrity was verified using an Agilent 2100 bioanalyzer (Agilent Technologies).

Total RNA (100 ng) was directly labeled with cyanine 3-CTP (Cy3), without fractionation or amplification, using an Agilent protocol that produces precise and accurate measurements spanning a linear dynamic range from 0.2 amol to 2 fmol of input miRNA. Each (100 ng) of 4 total RNAs from cancer tissue samples and a mixture of total RNAs (100 ng) extracted from normal epithelial tissues were competitively hybridized to a miRNA array (Agilent Microarray Design ID = 014947, Early Access version) containing 455 miRNAs, according to the manufacturer's protocol (27). A list of miRNAs contained in the array is available from version 8.2 of the Sanger miRNA database (http://microrna.sanger.ac.uk).

Data analysis. The intensity of each hybridization signal was evaluated using Feature extraction Software (Agilent Technologies). Feature Extraction analysis examines multiple probes and multiple features per probe and studies the measurements and errors for each miRNA. The observed values were imported into GeneSpring GX version 7.3 (Agilent Technologies). Generated miRNA profiles were normalized to the amount of input total RNA. A miRNA was designated overexpressed if expression was >2.6-fold compared to normal epithelial rectal tissues in all 4 clinical colorectal cancer samples (Table I).

MiRNA real-time RT-PCR. MiR-31-, *miR-183-*, *miR-17-5p-*, *miR-18a*, *miR-20a-*, *miR-92-* and *RNU6B* (internal control)-specific cDNAs were synthesized from total RNAs extracted from a maximum of 69 paired clinical samples using gene-specific primers according to *Taq*Man MicroRNA assays (Applied Biosystems). Reverse transcriptase reactions contained 10 ng of total RNA, 50 nM stem-loop RT primer, 1X RT buffer, 0.25 mM each of dNTPs, 3.33 U/μl MultiScribe reverse transcriptase and 0.25 U/μl RNase Inhibitor. The 7.5 μl reaction volumes were incubated in a 96-well plate in a Bio-Rad iCycler (Bio-Rad Laboratories) for 30 min at 16˚C, 30 min at 42˚C, 5 min at 85˚C and were then held at 4˚C.

Table I. Clinicopathological data in 4 colorectal cancers for microRNA microarray.

Case	1	2	3	4
Age	42	65	68	67
Gender	Female	Male	Male	Female
Tumor size (cm)	3.5x3.5	4.1x3.5	2.5x2.5	3.5x2.5
Location	S	Rb	S	S
Histological type	Well	Well	Well	Well
Lymph node metastasis	(+)	(+)	(-)	(-)
Lymphatic invasion	(+)	(-)	(-)	(-)
Venous invasion	(+)	(+)	(-)	(-)
Depth	se	se	sm	ss
Peritoneal dissemination	(-)	(-)	(-)	(-)
Liver metastasis	(-)	(-)	(-)	(-)
Stage	IIIa	IIIa	I	II
Outcome	Alive	Death	Alive	Alive

S, sigmoid colon; Rb, rectum (below the peritoneal reflection); well, well-differentiated adenocarcinoma; sm, submucosa; ss, subserosa and se, serosa.

Real-time PCR was performed using an Applied Biosystems 7500 real-time PCR system. Each 10 μl PCR volume included 0.67 μl RT products, 1X *Taq*Man Universal PCR master mix and 1 μl of primers and probe mix from each *Taq*Man microRNA assay. The reactions were incubated in 96-well optical plates at 95˚C for 10 min, followed by 45 cycles of 95˚C for 15 sec and 60˚C for 10 min. The relative expression of each miRNA was calculated using the $2^{-\Delta\Delta Ct}$ method, with the ratio of the median expression sample among all tumor samples/all non-tumor samples being used as the calibrator. The expression level of each miRNA was normalized to *RNU6B* expression.

Statistical analysis. Biostatistical analyses were performed with JMP 5.0.1a for Windows software (SAS Institute). Possible differences between groups were analyzed using the Student's t-test. Survival curves were obtained by the Kaplan-Meier method (28); comparison between curves was made by log-rank test. A probability level of 0.05 was chosen for statistical significance.

Results

Identification using miRNA array analysis of miRNAs that are overexpressed in clinical colorectal cancer. To investigate the differential expression of miRNAs in human colorectal cancers, array-based miRNA profiling of human colorectal

Table II. Twenty-one miRNAs up-regulated in clinical human colorectal cancers by miRNA array analysis.[a]

MicroRNA	Cases	Fold change
hsa-miR-31	4/4	179.29
hsa-miR-18b	4/4	175.71
hsa-miR-30e-3p	4/4	71.82
hsa-miR-220	4/4	51.05
hsa-miR-570	4/4	35.25
hsa-miR-302b	4/4	31.51
hsa-miR-302a	4/4	27.81
hsa-miR-183	4/4	18.94
hsa-miR-224	4/4	14.46
hsa-miR-18a	4/4	10.49
hsa-miR-95	4/4	10.13
hsa-miR-7	4/4	7.96
hsa-miR-182	4/4	5.63
hsa-miR-17-5p	4/4	4.83
hsa-miR-550	4/4	4.74
hsa-miR-196b	4/4	4.61
hsa-miR-181d	4/4	4.42
hsa-miR-20a	4/4	4.38
hsa-miR-92	4/4	4.14
hsa-miR-493-3p	4/4	4.07
hsa-miR-29a	4/4	3.75

[a]Fold change of miRNA expression was calculated relative to normal colorectal epithelial tissues. The bolded miRNAs signify those that were analysed by real-time RT-PCR.

cancer was performed. Out of 455 human miRNAs assayed, 21 were identified that had higher expression levels in colorectal cancer tissues than in normal epithelial tissues (Table II). *MiR-31* was the most up-regulated miRNA in the colorectal cancer tissues analyzed. Among the up-regulated miRNAs, *miR-17-5p*, *miR-18a*, *miR-20a* and *miR-92* are included in the *miR-17-92* cluster.

Real-time RT-PCR analysis of mature miRNAs. A maximum of 69 paired clinical samples were analyzed by real-time RT-PCR to quantify the expression of six up-regulated miRNAs (*miR-31*, *miR-183*, *miR-17-5p*, *miR-18a*, *miR-20a* and *miR-92*). The mean expression levels of *miR-31*, *miR-183*, *miR-17-5p*, *miR-18a*, *miR-20a* and *miR-92* were higher in tumor than in non-tumor tissues ($P<0.05$, Fig. 1). The percentages of cases in which the expression levels of *miR-31*, *miR-183*, *miR-17-5p*, *miR-18a*, *miR-20a* and *miR-92* were higher in tumor than in non-tumor tissues, were 63.8, 83.9, 71.6, 76.9, 77.6 and 66.7%, respectively (Table III).

We then investigated the expression levels of *miR-143* and *miR-145*, which were reported as down-regulated miRNAs in colorectal cancer (21,22,25,29). The mean expression levels of *miR-143* and *miR-145* were lower in tumor than in non-tumor tissues ($P<0.05$, Fig. 2). The percentages of cases in which the expression levels of *miR-143* and *miR-145* were lower in tumor than in non-tumor tissues, were 72.1 and 68.2%, respectively (Table III).

High miR-18a expression correlates with poor prognosis. We next surveyed the relationship between the expression of *miR-18a* and prognosis in colorectal cancer patients. Based upon the mean expression level of *miR-18a*, 65 clinical cases were divided into two groups: high *miR-18a* expression (n=21) and low *miR-18a* expression (n=44). The *miR-18a* overexpression group tended to have a poorer clinical prognosis than the low expression group ($P=0.07$; Fig. 3).

Table III. The expression level of up-regulated and down-regulated miRNAs were surveyed on paired clinical samples of colorectal cancer by real-time RT-PCR.

Up-regulated miRNAs	*miR-31*	*miR-183*	*miR-17-5p*	*miR-18a*	*miR-20a*	*miR-92*
Up-regulated/all cases (%)	44/69 (63.8)	52/62 (83.9)	48/67 (71.6)	50/65 (76.9)	52/67 (77.6)	46/69 (66.7)
Mean expression level in tumor (mean ± SD)	3.80±8.50	2.08±2.92	2.34±3.12	1.97±3.08	1.38±1.59	1.84±2.07
Mean expression level in non-tumor (mean ± SD)	0.70±0.8	0.39±0.38	1.01±1.27	0.86±1.23	0.48±0.55	1.03±0.83
Down-regulated miRNAs	***miR-143***	***miR-145***				
Down-regulated/all cases (%)	31/43 (72.1)	30/44 (68.2)				
Mean expression level in tumor (mean ± SD)	0.87±1.06	0.96±1.30				
Mean expression level in non-tumor (mean ± SD)	1.58±1.74	1.70±1.75				

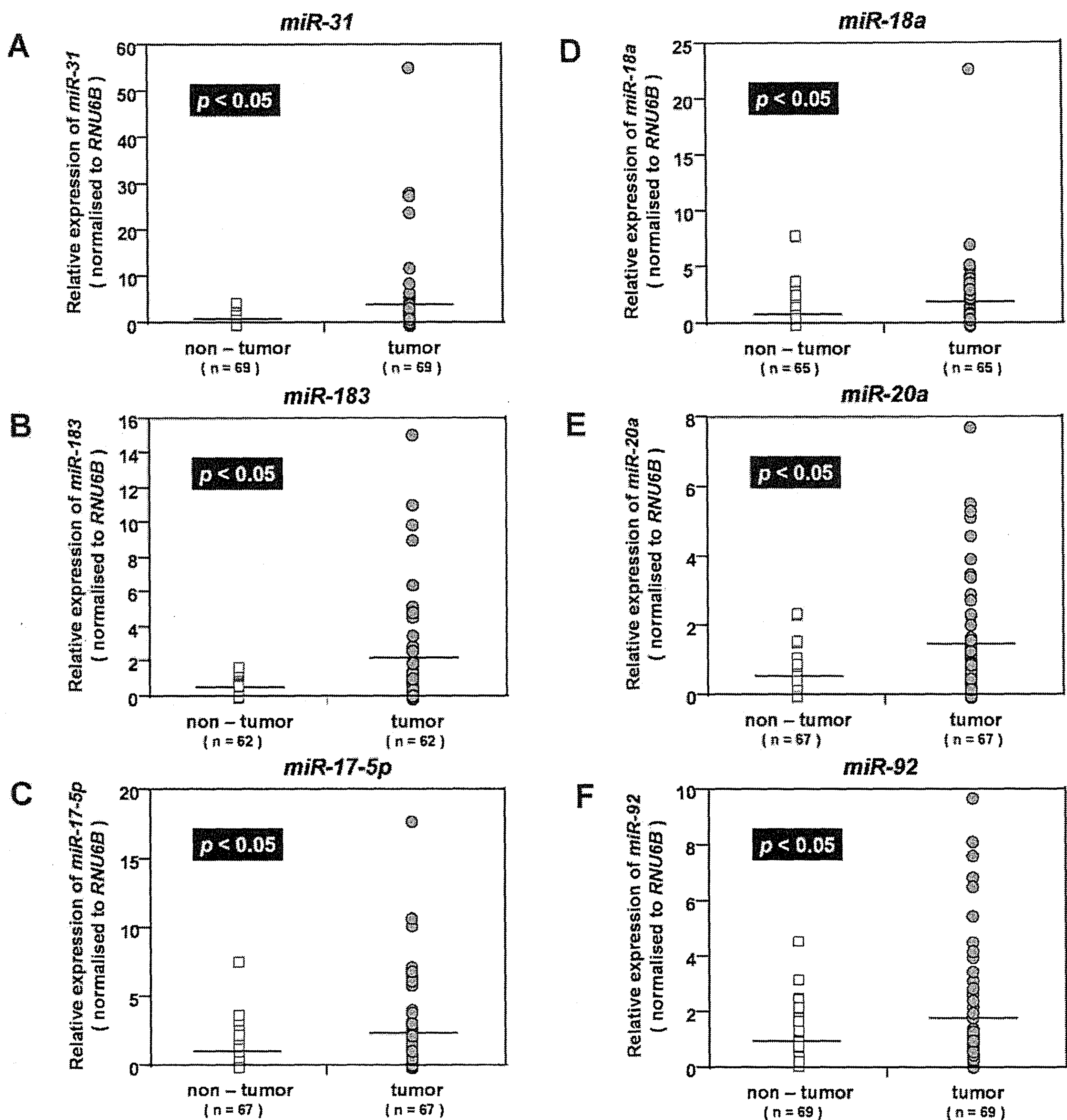

Figure 1. Part 1.

Discussion

Because of the large amount of evidence indicating that miRNAs are involved in carcinogenesis (15-20), it is important to identify colorectal cancer-related miRNA profiles by comprehensive analysis to increase understanding of colorectal cancer biology. In this study, a miRNA array with 455 known human miRNAs was applied to clinical samples of colorectal cancers to identify cancer-associated miRNAs. As a result, 21 cancer-related miRNAs were identified that were overexpressed in colorectal cancer tissues compared to normal colorectal epithelial tissues (Table II).

MiRNA expression profiling demonstrated that *miR-31* expression was the highest among the 21 overexpressed miRNAs seen in the colorectal cancer samples that were assayed (Table II). In addition, real-time RT-PCR analysis of 69 clinical colorectal cancers showed that *miR-31* expression was significantly higher in cancer than in normal tissues (Fig. 1A and a). Bandres *et al* surveyed the expression of 156 mature miRNAs in 16 colorectal cancer cell lines and 12 matched pairs of tumor and non-tumor tissues by real-time PCR and reported that *miR-31* was one of the seven overexpressed miRNAs and was associated with tumor stages of colorectal cancer (24). Slaby *et al* reported that the

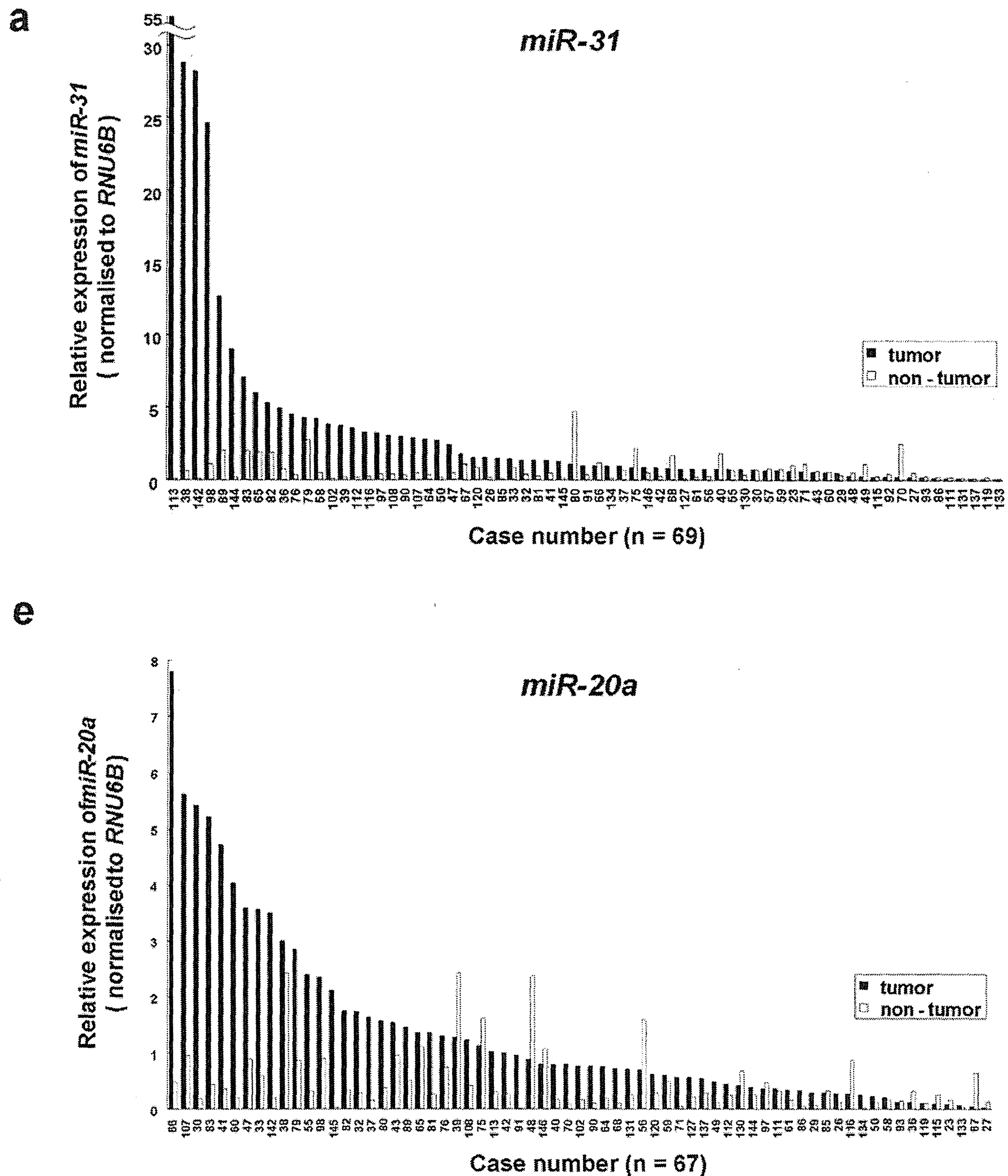

Figure 1. Part 2. Real-time RT-PCR analysis of 6 up-regulated miRNAs in tumor and non-tumor samples from colorectal cancer cases. Expression values of miRNAs were shown as the expression ratio of each miRNA to *RNU6B*. (A, a) *miR-31* expression; (B) *miR-183* expression; (C) *miR-17-5p* expression; (D) *miR-18a* expression; (E, e) *miR-20a* expression; (F) *miR-92* expression. Horizontal lines indicate the mean values of tumor and non-tumor samples.

expression of *miR-31* was up-regulated in 29 primary colorectal cancers (29). These studies suggest that *miR-31* is one of the important colorectal cancer-related miRNAs. Up-regulated miRNAs listed by Bandres *et al* included *miR-183* and *miR-20a*, which is consistent with our data that *miR-183* and *miR-20a* were overexpressed in our colorectal cancer samples by both miRNA array and real-time RT-PCR analysis (Table II, Fig. 1B, E and e).

We demonstrated that expression of *miR-17-5p*, *miR-18a*, *miR-20a* and *miR-92*, which are cleavage products of the *miR-17-92* cluster, were individually up-regulated in our miRNA expression profiles and that by real-time RT-PCR analysis, their expression in clinical sample cancer tissues were also significantly higher than in normal tissues (Table II, Fig. 1C-F). The *miR-17-92* cluster is comprised of 6 miRNAs (*miR-17-5p*, *miR-18a*, *miR-19a*, *miR-19b-1*, *miR-20a* and *miR-92*) (30). The *miR-17-92* cluster is overexpressed in malignant lymphoma cell lines and lung cancers (31,32) and cooperates with c-MYC to accelerate tumor development (31). The introduction of *miR-17-92* can enhance the growth

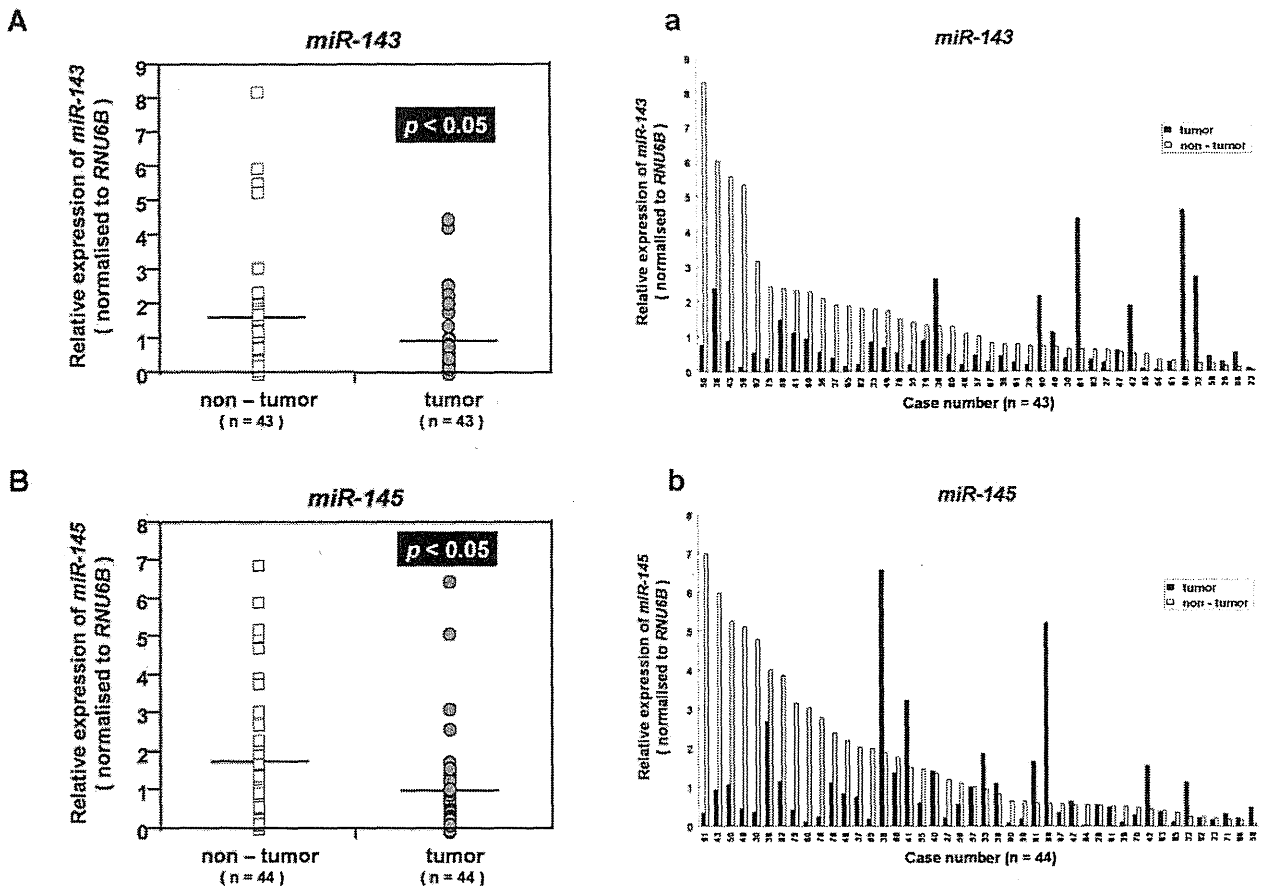

Figure 2. Real-time RT-PCR analysis of 2 down-regulated miRNAs in tumor and non-tumor samples from colorectal cancer cases. (A, a) *miR-143* expression; (B, b) *miR-145* expression. Horizontal lines indicate the mean values of tumor and non-tumor samples.

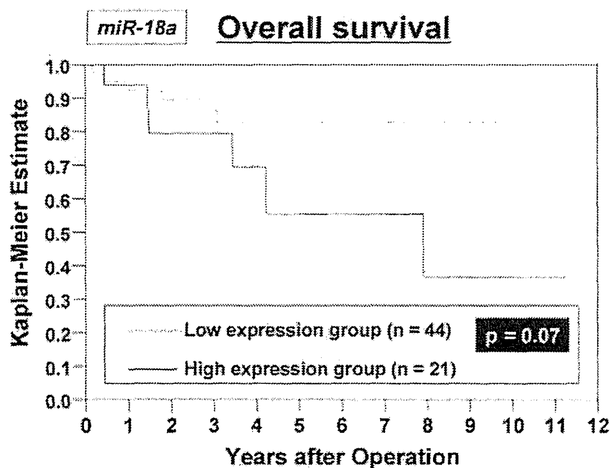

Figure 3. Survival curves of colorectal cancer patients according to *miR-18a* expression status. High expression group (n=21): *miR-18a* > mean value; low expression group (n=44): *miR-18a* < mean value. By the log-rank test, patients with high *miR-18a* expression tended to have poorer prognoses than those with low *miR-18a* expression (P=0.07).

property of lung cancer cells *in vitro* (32). Additional evidence indicates that *miR-17-92* can be a tumor angiogenesis mediator (33) and affects the expression of the members of the E2F family of oncogenic transcription factors (34-36). These studies suggest that the *miR-17-92* polycistron may be the most prominent oncogenic miRNA cluster. Most recently, Ventura *et al* reported a link between the oncogenic properties of *miR-17-92* and its functions during B cell lymphopoiesis and lung development (37).

In clinical colorectal cancers, He *et al* reported that the level of *miR-17-92* pri-miRNA was up-regulated in 15% of tested samples and showed >5-fold up-regulation compared to corresponding normal tissues by real-time quantitative PCR (31). In this study, the percentages of cases with >5-fold expression levels of *miR-17-5p*, *miR-18a*, *miR-20a* and *miR-92* in clinical colorectal cancer tissues were 29.2, 19.4, 31.3 and 11.6%, respectively. Volinia *et al*, using prediction analysis of microarrays (PAM), reported that 21 miRNAs were overexpressed in 46 colorectal cancer samples compared to 8 normal colorectal tissues (23) and that elevated expression levels of *miR-17-5p* and *miR-20a* were found by miRNA expression profiling. Schetter *et al* reported that 27 miRNAs were overexpressed in miRNA array expression profiling of 84 colorectal tumor and paired non-tumorous tissues, which included *miR-17-5p*, *miR-20a* and *miR-92* (26). Matsubara *et al* showed that inhibition of *miR-17-5p* and *miR-20a* expression by antisense oligonucleotides could selectively induce apoptosis in lung cancer cells that overexpressed *miR-17-92* (38). To our knowledge, there have been no studies

published that elucidate the biology of *miR-17-5p* and *miR-20a* in human colorectal cancer.

Recently, *miR-200c* (39) and *miR-21* (26) expression were reported to be associated with poor survival in colorectal cancer patients. In this study, we demonstrated that the *miR-18a* high expression group tended to have a poorer prognosis than the low expression group (P=0.07: Fig. 3) and we believe that *miR-18a* expression can be used as a prognostic factor in predicting survival of postoperative colorectal cancer patients.

The expression of *miR-143* and *miR-145* are down-regulated in colorectal tumors and their *in vitro* transfection into human colon cancer cell lines (DLD-1, SW480) led to growth inhibition (21,22,25). In our study, real-time RT-PCR analysis showed that expression of *miR-143* and *miR-145* were significantly lower in 43 and 44 clinical colorectal cancers, respectively, than in normal tissues (P<0.05: Fig. 2).

In conclusion, this study identified 21 up-regulated miRNAs in human colorectal cancers. *MiR-31*, *miR-183*, *miR-18a*, *miR-17-5p*, *miR-20a* and *miR-92* were significantly overexpressed in cancer compared to normal tissues. The advent of miRNA research may lead to possible applications to molecular diagnostics and prognostics in colorectal cancer. More study is required to clarify the precise contributions of miRNAs to colorectal cancer progression.

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Expression of *uPAR* mRNA in peripheral blood is a favourite marker for metastasis in gastric cancer cases

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Urokinase-type plasminogen activator receptor (*uPAR*) plays a central role in the plasminogen activation cascade and participates in extracellular matrix degradation, cell migration and invasion. We evaluated the expression level of *uPAR* mRNA and the presence of isolated tumour cells (ITCs) in bone marrow (BM) and peripheral blood (PB) in gastric cancer patients and clarified its clinical significance. We assessed specific *uPAR* mRNA expression by quantitative real-time reverse transcriptase- polymerase chain reaction (RT–PCR) in BM and PB in 846 gastric cancer patients as well as three epithelial cell markers, carcinoembryonic antigen (*CEA*), cytokeratin (*CK*)-*19* and *CK-7*. The *uPAR* mRNA expression in bone marrow and peripheral blood expressed significantly higher than normal controls ($P<0.0001$). The *uPAR* mRNA in BM showed concordant expression with the depth of tumour invasion, distant metastasis, and the postoperative recurrence ($P=0.015$, 0.044 and 0.010, respectively); whereas in PB, we observed more intimate significant association between *uPAR* expression and clinicopathologic variables, such as depth of tumour invasion, the distant metastasis, the venous invasion and the clinical stage ($P=0.009$, 0.002, 0.039 and 0.008, respectively). In addition, the *uPAR* mRNA expression in PB was an independent prognostic factor for distant metastasis by multivariate analysis. We disclosed that it was possible to identify high-risk patients for distant metastasis by measuring *uPAR* mRNA especially in peripheral blood at the timing of operation in gastric cancer patients.
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The presence of isolated tumour cells (ITCs) in bone marrow (BM) and peripheral blood (PB) is missed by conventional imaging system, and ITCs expected to be a determinant factor of subsequent formation of micrometastasis. The search for ITCs in patients with curatively resected tumours is of considerable importance, because early dissemination of tumour cells is one of the leading causes of relapse at the distant site and of death from cancer (Hellman, 1997). In spite of large number of studies to determine the clinicopathologic significance of ITCs in human solid carcinomas, much efforts have been made and found no definitive evidence to conclude these controversial issues, such as the way to identify ITCs and the appropriate biologic marker to predict the metastatic ability of gastric cancer cells (Heiss *et al*, 1995, 1997, 2002; Jauch *et al*, 1996; Hardingham *et al*, 2000; Lecomte *et al*, 2002; Natsugoe *et al*, 2003; Ikeguchi and Kaibara, 2005).

To identify ITCs in BM or PB, a bunch of molecular targets, such as CEA, CK7, CK18, CK18, CK19 and MAGE families have been applied and examined whether those genes were applicable to detect ITCs. Above all candidates, *CEA* and cytokeratin *(CK)*, epithelial cell surface markers were frequently used to be applied to detect ITCs instead of the direct detection of cancer cells in those ITC studies. However, our recent study disclosed that ITCs in BM and PB from gastric cancer could not be specified by the presence of CEA and/or CKs by RT–PCR, because those genes were detected ubiquitously among stages of 810 patients of gastric cancer. Therefore, we must identify a favourite marker to detect ITCs specifically as well as to predict metastasis precisely. In the current study, we focused on urokinase-type plasminogen activator receptor (*uPAR*) gene as a target molecule to detect isolated tumour cells in blood and bone marrow. This is because that in gastric cancer, several reports showed clearly that uPAR expression in bone marrow (BM) is one of the useful prognostic marker by immunohistochemistry (Heiss *et al*, 1995, 2002; Allgayer *et al*, 1997). However, there are no earlier reports of the clinical significance of the gene expression level of *uPAR* with quantitative RT-PCR assay that enabled us to examine objectively and repeatedly. Therefore, the aim of the current study was to evaluate the expressions of *uPAR* mRNA in bone marrow and peripheral blood in more than 800 cases of gastric cancer and to define its clinicopathologic and prognostic significance in gastric cancer patients.

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