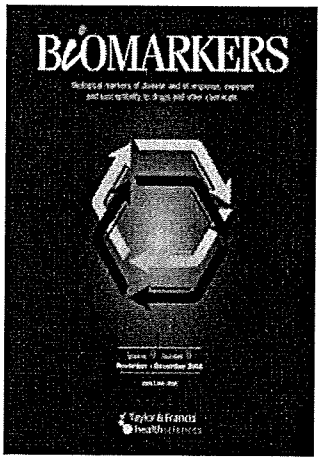

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MicroRNA-500 as a potential diagnostic marker for hepatocellular carcinoma

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ORIGINAL ARTICLE

MicroRNA-500 as a potential diagnostic marker for hepatocellular carcinoma

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Abstract

We identified that microRNA expression changed dynamically during liver development and found that miR-500 is an oncofetal miRNA in liver cancer. miR-500 was abundantly expressed in several human liver cancer cell lines and 45% of human hepatocellular carcinoma (HCC) tissue. Most importantly, an increased amount of miR-500 was found in the sera of the HCC patients. In fact, miR-500 levels in sera of the HCC patients returned to normal after the surgical treatment in three HCC patients. Our findings reveal that diverse changes of miRNAs occur during liver development and, one of these, miR-500 is an oncofetal miRNA relevant to the diagnosis of human HCC.

Keywords: *miRNA; miR-500; hepatocellular carcinoma, liver development, diagnosis*

Introduction

MicroRNAs (miRNAs) are small RNA molecules of 21–25 nt that have the potential to a play central role in physiological and pathological processes, including cell differentiation, apoptosis and oncogenesis (Ambros 2004, Esquela-Kerscher et al. 2006). The biogenesis of miRNAs involves nucleolytic processing of precursor transcripts, which are transcribed from different genomic locations as long primary transcripts (pri-miRNA) by RNA polymerase II in the nucleus (Lee et al. 2004). Pri-miRNAs are processed by the RNase-III family of an enzyme, Drosha, to a ~70 nt precursor called the pre-miRNA. The pre-miRNA is exported to the cytoplasm by Exportin-5 and then cleaved in the cytoplasm by Dicer to ~22 nt double-strand mature miRNA (Han et al. 2006, Lund et al. 2004, Ketting et al. 2001). A single strand of the mature miRNA is assembled into effecter complexes called miRNPs (miRNA-containing ribonucleoprotein particles), which share a considerable amount of similarity with an RNA-induced silencing complex (RISC) (Nelson et al. 2004). They induce gene suppression post-transcriptionally by inducing mRNA degradation or by regulating the translational efficiency of mRNA (Bartel 2004).

Several reports have shown the importance of miRNA functions in tissue development. More recent reports, in particular those regarding comprehensive microRNA profiling analysis, have shown that miRNAs are expressed in a tissue-specific manner and their expression altered

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in the process of development, such as cardiogenesis and haematopoiesis (Chen et al. 2006, 2004). For example, miR-1, which is expressed specifically in cardiac and skeletal muscle, is essential for cardiac morphogenesis and conduction (Zhao et al. 2007). Another study showed that miR-181a regulates intrinsic antigen sensitivity during T-cell development (Li et al. 2007). Another important aspect of miRNA study is the association of its gene targets and disease, which have been investigated by many researchers. Mir-17-92 polycistron has been designated as oncomiR-1 (He et al. 2005), and let-7 family miRNAs and miR-34 function as tumour suppressors (Johnson et al. 2005, Yu F et al. 2007, He et al. 2007); moreover, a number of studies have given evidence that several miRNAs are associated with carcinogenesis and regulate the expression of cancer-related genes.

Although emerging evidence suggests that several miRNAs are involved in the process of liver development (Esau et al. 2006, Fu et al. 2005, Gramantieri et al. 2007), the roles of miRNAs in hepatogenesis and their possible relation to hepatocarcinogenesis have not been thoroughly examined. In this study, to investigate liver development from the biological aspects of microRNA, we performed a mouse miRNA microarray carrying 340 miRNA probes. We report that some of these miRNAs are strongly expressed, and that dynamic changes in their expression profile are observed in the process of liver development. We also show that miR-500 is an oncofetal miRNA, which is highly expressed in fetal liver, more than in adult normal liver, and aberrantly expressed in hepatocellular carcinoma (HCC) tissue. Thus, dynamic miRNA regulation is an important feature as an oncofetal non-coding small RNA relevant to the diagnosis of human liver cancer.

Materials and methods

RNA extraction

C57BL/6J mice were used in this study. Total RNA from mouse liver tissues (embryo (E) 14, E16, E18, neonate and adult), *in vitro* fetal hepatocyte cultured samples (days 0, 1, 3, 5 and 7), and liver cancer cell lines (HepG2, Huh-7, JHH-7, Alexander, Li-7, and Hep3B) were extracted using the mirVana™ miRNA Isolation Kit (Ambion, Tokyo, Japan). Animal experiments in the present study were performed in compliance with the guidelines of the Institute for Laboratory Animal Research, National Cancer Center Research Institute.

Locked nucleic acid (LNA)-based miRNA microarray

The miRCURY™ LNA array version 8.0, which contains capture probes targeting all human, mouse and rat miRNA listed in the miRBASE version 8.0, was applied to detect the expression of mouse miRNA (Exiqon, Vedbaek, Denmark). Total RNA samples were collected from fetal (E14, 16 and 18), neonate and adult (8-week-old) mice ($n = 7$–10). Total RNA samples (2000 ng) from liver tissue and reference (Universal control, which is made from mouse tissue mixtures) were labelled with the Hy3™ and Hy5™ fluorescent stain, respectively, using the miRCURY™ LNA Array labelling kit according to the procedure described by the manufacturer (Exiqon). Hybridisation and normalisation were performed according to the miRCURY™ LNA array manual, and image analysis of the miRCURY™ LNA array microarray slides was acquired using an Agilent Technologies Microarray Scanner and Agilent Feature Extraction 9.1 (Agilent Technologies, Tokyo, Japan). A hierarchical cluster was produced from microarray data using a Euclidean distance calculation based on Ward's methods by GenMaths software (Applied Maths). All the miRNA microarray data are shown in Supplementary Table 1 (see the online version of this article).

Cell culture

Liver cancer cell lines (HepG2, Huh-7, JHH-7, Alexander, Li-7 and Hep3B) were cultured in liquid culture with Dulbecco's modified eagle medium (DMEM; GIBCO Laboratories, Grand Island, NY, USA) supplemented with heat-inactivated 10% fetal bovine serum (FBS; Equitech-Bio, Kerrville, TX, USA) and a 1% antibiotic antimycotic solution (Invitrogen, Tokyo, Japan). The cells were maintained *in vitro* at 37°C in a humidified atmosphere with 5% CO_2.

Patients and RNA specimens

Liver tissues were obtained surgically with informed consent from patients at the National Cancer Center Hospital (Tokyo, Japan). The study was approved by the Institutional Review Board of the National Cancer Center Research Institute. Liver tissue total RNAs were extracted from 40 HCC patients and their associated non-cancerous tissue. The clinical data and pathological diagnosis are summarized in Supplementary Table 2 (see the online version of this article).

Real-time polymerase chain reaction

Total RNAs of approximately 100 ng were reverse-transcribed using the Taqman miRNA reverse transcription kit (Applied Biosystems, Tokyo, Japan). Real-time quantitative polymerase chain reaction (PCR) amplification of the cDNA template was done using Taqman Universal PCR Master Mix (Applied Biosystems) in

an ABI PRISM 7300 (Applied Biosystems). The PCR conditions were 50°C for 2 min and 95°C for 10 min followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. Taqman probes for human and mouse miRNA were used to assess the expression levels of miRNA (mmu-miR-101b, ID 4373159; mmu-122a, ID 4373151; mmu-miR-142-5p, ID 4373135; mmu-miR-223, ID 4373075; mmu-miR-451, ID 4373360; has-miR-346, ID 4373038; has-miR-500, ID 4373225; Applied Biosystems). The expression levels were normalised against U6 (RNU6B, ID 4373381; Applied Biosystems) or total RNA volume.

RNA isolation from human serum samples

Whole blood samples were obtained from patients with HCC at the Kyoto University (Kyoto, Japan). All of the donors or their guardians provided written consent and ethics permission was obtained for the use of all samples. Blood samples were taken before and after completion of surgery. Serum samples were stored at -80°C until analysis. For serum RNA isolation, total RNA was isolated using Isogen (Nippon Gene, Japan), according to the manufacturer's instructions.

Measurement of serum miRNA levels by using TaqMan qRT-PCR assays

A fixed volume of 5 µl of RNA solution (14 ng) was used as input into the reverse transcription reaction. Input RNA was reverse transcribed using the TaqMan miRNA Reverse Transcription Kit and miRNA-specific stem-loop primers (Applied BioSystems) in a small-scale reverse transcription reaction (comprising 2 µl of H_2O, 1 µl of 10x reverse-transcription buffer, 0.2 µl of RNase inhibitor (20 units ml^{-1}), 0.1 µl of 100 mM dNTPs, 0.7 µl of Multiscribe reverse transcriptase and 5 µl of input RNA), using a Tetrad2 Peltier Thermal Cycler (BioRad, Tokyo, Japan) at 16°C for 30 min, 42°C for 30 min and 85°C for 5 min. Teverse transcription product (4.75 µl) was combined with 5.25 µl of PCR assay reagents (comprising 5 µl of TaqMan 2x Universal PCR Master Mix, No AmpErase UNG and 0.25 µl of TaqMan miRNA assay) to generate a PCR of 10.0 µl of total volume. Real-time PCR was performed as described above. Serum levels of miR-16 were measured as internal normalisation control as they were not significantly different between controls and patients in prostate cancer and colorectal cancer (Mitchell et al. 2008).

Statistical analysis

The results are given as mean ± SD. The Student's *t*-test was performed for statistical evaluation; $p<0.05$ or $p<0.001$ was considered significant.

Table 1. MicroRNAs (miRNA) abundantly expressed in liver development.

Liver stage	MiRNA name[a]
E14	miR-122a, miR-142-3p, miR-142-5p, miR-144, miR-223, miR-346, miR-374-5p, miR-451, miR-486, miR-500
E16	miR-101b, miR-122a, miR-142-3p, miR-142-5p, miR-144, miR-223, miR-295, miR-346, miR-367, miR-374-5p, miR-451, miR-464, miR-471, miR-486, miR-500, miR-547
E18	miR-101b, miR-122a, miR-142-3p, miR-142-5p, miR-144, miR-223, miR-324-3p, miR-374-5p, miR-451, miR-486
Neonate	miR-101b, miR-122a, miR-142-3p, miR-142-5p, miR-144, miR-223, miR-463
Adult	miR-21, miR-22, miR-29a, miR-29b, miR-29c, miR-101a, miR-101b, miR-122a, miR-126-5p, miR-192, miR-374-5p

[a]miRNAs are listed in ascending order. E, embryo.

Results

Analysis of the global expression levels of miRNA in the process of liver development by LNA-based miRNA microarray

To examine how the expression profile of miRNA changed in the process of mouse liver development, we performed an LNA-based miRNA microarray at different developmental stages. Total RNAs from E14, 16, 18, neonate and adult liver were isolated and labelled with Hy3, and total RNAs of universal control consisted of several tissue mixtures labelled with Hy5 as a common reference. After normalisation of the miRNA expression, the number of high- and low-expressed miRNAs at different time stages was counted. High-expressed miRNA represents twofold or more upregulated miRNA, and low-expressed miRNA represents twofold or more downregulated miRNA, when compared with an average expression level of all miRNAs (see Supplementary Figure 1 in the online version of this article). Throughout all developmental stages of the liver, most of the miRNA expression levels were classified as low-expressed miRNA; in contrast, the number of high-expressed miRNAs was quite limited and are listed in Table 1. These data indicated that expression levels of the general miRNAs were very low and that a limited number of miRNAs were highly expressed in mouse liver development.

Differential expression patterns of miRNAs in the process of mouse liver development

To determine differentially expressed miRNA and to quantify the expression changes in the process of liver

development, hierarchical unsupervised clustering analysis was performed using microarray data of E14, 16, 18, neonate and adult mouse liver. The case cluster analysis of the microarray data indicated a similarity of clusters from the viewpoint of the expression pattern between E14 and E16 fetal liver and between neonate and adult liver (Figure 1), indicating that the miRNA expressions changed depending on the developmental stage. These results indicated that expression of most of the miRNAs was regulated precisely in the process of liver development.

The expression pattern of miRNA selected from highly expressed miRNAs (Table 1) was verified by real-time PCR to show the accuracy of miRNA expression acquired from the microarray analysis. The left panels of Figure 2 present the results of microarray analysis for five miRNAs (miR-101b, miR-122a, miR-142-5p, miR-223 and miR-451). Expressions of miR-101b and miR-122a were low at the early stage of liver development and were upregulated during maturation. In contrast, expressions of miR-142-5p, miR-223 and miR-451 were high at the early stage of liver development and were already known as miRNAs expressed in haematopoietic cells (Chen et al. 2004, Zhan et al 2007, Johnnidis et al 2008). The right panels of Figure 2 are the results of real-time PCR for the same set of miRNAs. In comparison to the microarray results and the real-time PCR results, these data obtained from two different methods showed approximately similar expression patterns of miRNAs, confirming the validity of our microarray analysis.

Interestingly, miRNAs (miR-142-5p, miR-451 and miR-223) expressed in haematopoietic cells were highly expressed at the early stages (E14 and E16) and then gradually downregulated in the process of liver development (Figure 2). Because whole fetal liver is a haematopoietic organ and a large number of haematopoietic cells are contained there, this also indicated the accuracy of expression profiling of miRNA in the process of liver development by LNA-based microarray.

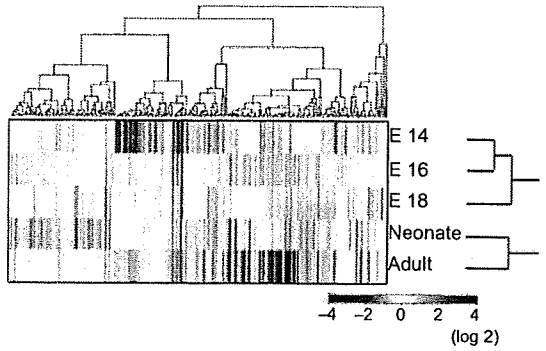

Figure 1. A global expression pattern of miRNA in the process of mouse liver development. The data were subjected to a hierarchical cluster analysis using a Euclidean distance calculation based on Ward's methods. The liver samples are aligned vertically: embryo (E) 14, E16, E18, neonate and adult. Samples were linked by the dendrogram shown on the right to highlight the similarity in their miRNA expression patterns. The expression profile of each miRNA is depicted in the respective row. The expressions of miRNA are linked by the dendrogram shown on the top to highlight the similarity in their expression patterns.

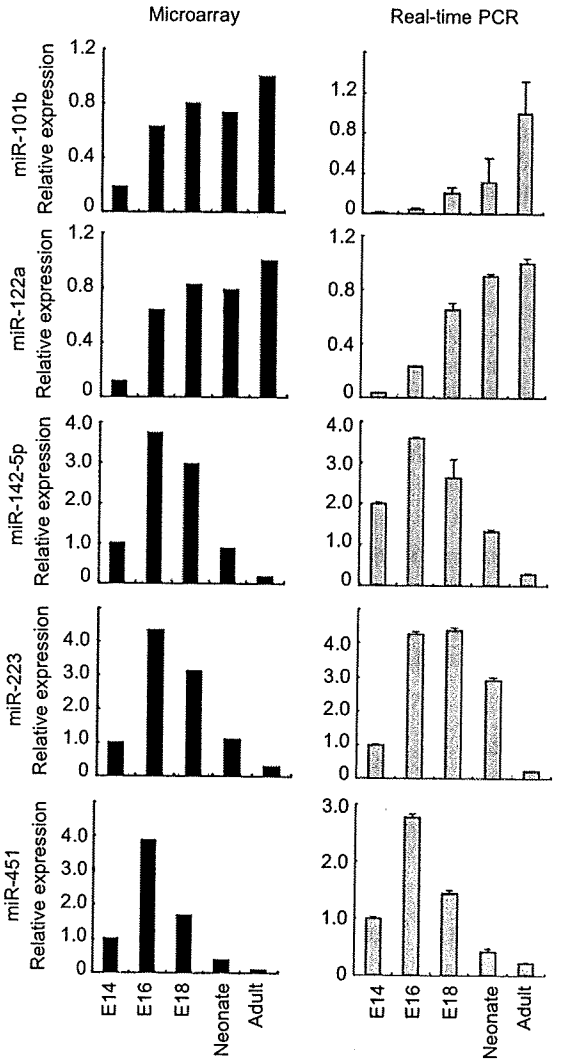

Figure 2. Differential expression of selected miRNA in mouse liver development by microarray and real-time polymerase chain reaction (PCR). miR-101b, miR-122a, miR-142-5p, miR-223 and miR-451 were selected from highly expressed miRNAs to confirm the expression levels of microarray analysis by real-time PCR. The left panels represent the miRNA expression levels by microarray analysis. The right panels represent the miRNA expression levels by real-time PCR. The expression profile is compared for mouse fetal (embryo (E) 14, E16 and E18), neonate and adult liver. In the graphs of miR-142-5p, miR-223 and miR-451, the expression level of E14 fetal liver is set to 1.0. Real-time PCR analyses were performed in triplicate and expression values are normalized with total RNA volume. Data are shown as mean ± SD.

Differential expression patterns of cancer-related miRNAs in the process of mouse liver development

Interestingly, when analysing the expression patterns of the hierarchical clustering data in detail, we found that the expression of several let-7 miRNA family known as 'tumour suppressor miRNA' was upregulated, and, in contrast, the expression of miRNAs known as 'potential oncogenes' which are involved in cell proliferation, was downregulated in the process of liver development. Therefore, to reveal the expression pattern of cancer-related miRNAs in the process of mouse liver development, the expression profile of 21 selected miRNAs (11 miRNAs as oncogenes and 10 miRNAs as tumour suppressors) is summarized in Figure 3. Many oncogenic miRNA expressions, such as those of miR-17-5p, miR-20, and miR-92, tended to decrease in the process of mouse liver development (Figure 3A). In contrast, except for let-7d* and let-7e, the expression pattern of the let-7 miRNA family was elevated in the process of liver development (Figure 3B). This study provides evidence that the expression of oncogenic miRNA is downregulated and that the expression of tumour suppressor miRNA is upregulated in the process of liver development.

Expression of miR-500 is high in human fetal liver

As reported above, the expression levels of oncogenic miRNAs were downregulated in liver development. We tried to identify new miRNA candidates that act as an oncogenic miRNA in the liver from the microarray data. As a first step toward the elucidation of the role of miRNAs in liver carcinogenesis, we focused on downregulated miRNAs during liver maturation, which are possibly related to cell proliferation; high expressions of miR-140, miR-346, miR-411, miR-470 and miR-500 were detected at an early stage (E14) of liver development and downregulated at the late developmental stages (E16 and E18) (Figure 4A). Among these, miR-500 and miR-346 expressions were remarkably downregulated during development; thus, we concentrated on miR-500 and miR-346, which could be expected to be a potential target relevant to fetal liver development to control the time and spatial expression of sets of mRNA.

In the next step, the occurrence of miR-500 and miR-346 was assessed in human fetal and adult liver. Real-time PCR analysis revealed that the expression of miR-500 in human fetal liver, but not that of miR-346, was significantly higher than that in normal adult liver (Figure 4B and Supplementary Figure 2A (see online version of this article)). Taken together, as miR-500 expression was downregulated in human adult liver, our data suggest that miR-500 is developmentally associated with human fetal hepatocyte specification and functions. The

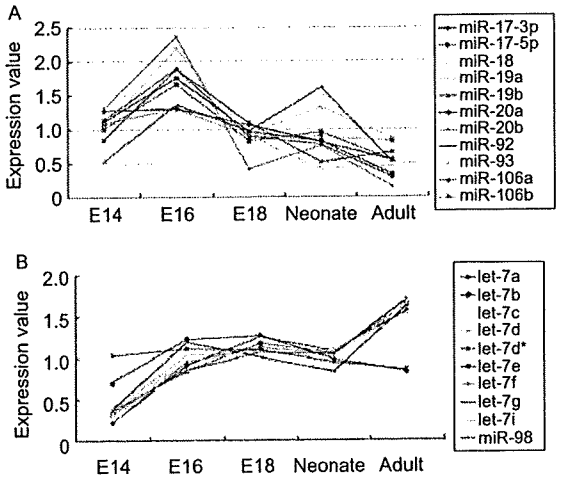

Figure 3. Expression patterns of cancer-related miRNAs in the process of mouse liver development. (A) Expression pattern by a microarray analysis (each sample: $n=7$–10) of miRNA that may act as an oncogene. (B) The expression pattern by the microarray analysis (each sample: $n=7$–10) of the let-7 family miRNAs functioned as a tumour suppressor. Expression levels are normalised by average expression value of each miRNA and shown in the graph.

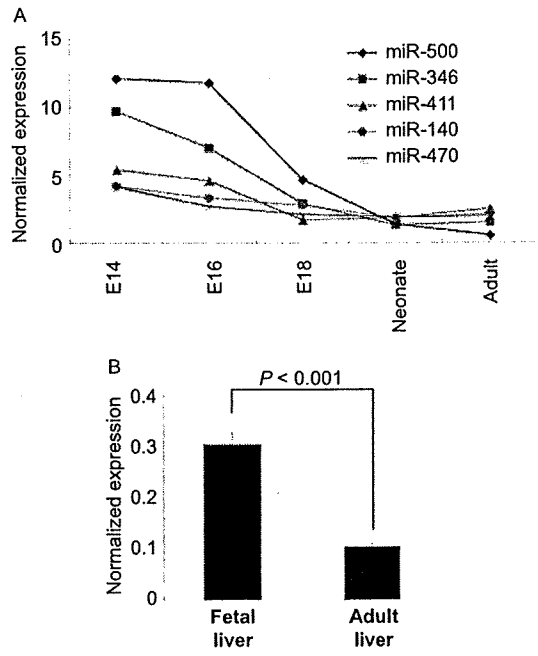

Figure 4. The expression of miR-500 is higher in the fetal stage than in the adult stage (A). The expression profile of miRNA decreased in the process of mouse liver development. Expression values are based on microarray data. (B) Expression of miR-500 in human fetal and adult liver. Real-time polymerase chain reaction analyses were performed in triplicate. Expression values are normalised with U6 snRNA value. The data represent the mean ± SD, $p<0.001$.

results of our ongoing knock-down analysis of miRNA in liver cancer cells will be presented in a future work.

Expression of miR-500 is high in human liver cancer

We next examined the expression level of miR-500 in six human liver cancer cell lines (JHH-7, Li-7, Huh-7, HepG2, Hep3B and Alexander) to assess whether miR-500 acts as an oncofetal miRNA and found that it increases 2.4- to 47.6-fold more in Alexander, JHH-7, HepG2, Huh-7 and Hep3B than in normal liver (Figure 5A); in contrast, no detectable amount of miR-500 was found in Li-7. On the other hand, the expression levels of miR-346 in the six liver cancer cell lines were not high (see Supplementary Figure 2B in the online version of this article). To evaluate the potential of miR-500 as an oncofetal miRNA, the expression levels of human miR-500 were analysed by real-time PCR in 40 pairs of malignant neoplasias of hepatocyte lineage (T) and adjacent non-tumorous tissue (NT). Differences in the miR-500 expression level were statistically significant ($p<0.001$) between T and NT (Figure 5B), but miR-346 expression was not significantly changed (see Supplementary Figure 2C in the online version of this article). Some of the samples exhibited remarkably high expression levels of miR-500, and 45% (18/40 patients) of the samples showed 1.2- to 8.6-fold higher upregulation in the cancerous samples than in each non-tumorous sample and

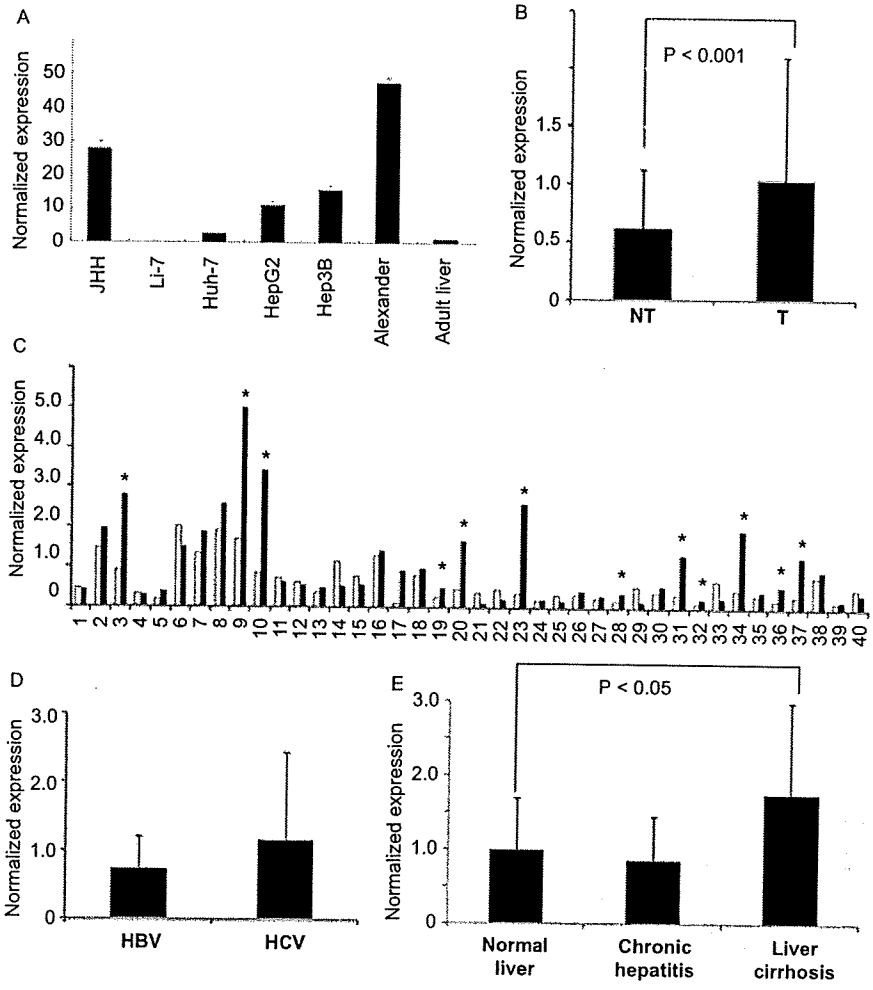

Figure 5. The expression of miR-500 is clearly upregulated in human liver cancer (A) miR-500 expression abundantly detected in liver cancer cell lines (JHH-7, Li-7, Huh-7, HepG2, Hep3B and Alexander). The expression level of normal liver is set to 1.0. The data represent the mean ± SD. (B) Forty pairs of hepatocellular carcinoma (HCC) patients (tumour (T) and non-tumour (NT)) were analysed by real-time polymerase chain reaction of human miR-500. The data represent the mean ± SD, $p<0.001$. (C) Expression levels of miR-500 in each patient (T and NT). Samples of 12 patients (*) showed twofold or more upregulation in HCC. (D) Expression levels of miR-500 in hepatitis B virus (HBV, $n=10$) and hepatitis C virus (HCV, $n=26$). (E) miR-500 expression was upregulated in liver cirrhosis ($n=17$) more than normal liver ($n=11$) and chronic hepatitis samples ($n=19$). The data represent the mean ± SD, $p<0.05$. Expression values are normalised with U6 snRNA value.

12 patients showed more than 2.0-fold higher expression (30%) (Figure 5C). Based on the clinical data and pathological diagnosis (see Supplementary Table 2 in the online version of this article), there is no significant difference in miR-500 expression between hepatitis virus B and C infection (Figure 5D). Importantly, significant difference in miR-500 expression was found between normal liver and liver cirrhosis samples, but not chronic hepatitis (Figure 5E), suggesting that miR-500 expression was upregulated during cirrhosis development. Thus, although only limited samples expressed miR-500 higher, miR-500 might be useful as a biomarker in the early stage of liver cancer.

Expression profiling of miR-500 in HCC patient serum

Recently, it has been reported that miRNAs are circulating in serum (Chim et al. 2008, Gilad et al. 2008) and tumour-derived miRNAs such as miR-155, miR-21, miR-15b, miR-16 and miR-24 are detected in the plasma and serum of tumour patients (Mitchell et al. 2008, Lawrie et al. 2008). In fact, an increased amount of miR-500 was found in the sera of three out of ten HCC patients, which means that liver cancer-specific miRNA such as miR-500 is circulating in the peripheral blood and can be a novel diagnostic marker. To determine whether or not serum levels of miR-500 truly reflect the presence of cancer in the HCC patients, the presence of miR-500 in the sera of three human HCC patients, post- and presurgical treatment, was also assessed. As can be seen in Figure 6, elevated serum levels of miR-500 in the three HCC patients were significantly reduced after surgery and returned to normal levels. These results expect that the miR-500s abundance profile in serum of the HCC patients might reflect physiological and/or pathological conditions.

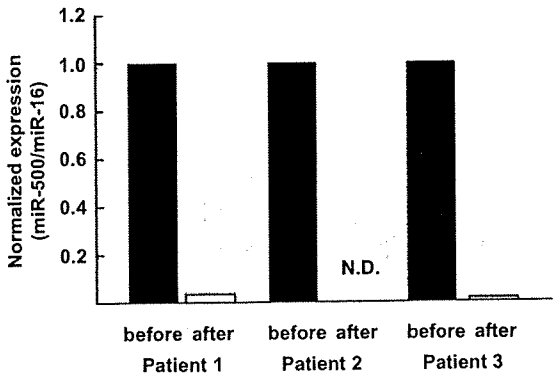

Figure 6. Serum levels of miR-500 in hepatocellular carcinoma (HCC) patients. Changes of serum levels of miR-500 in HCC patients ($n=3$) before (preoperation) and after (postoperation within 6 months) surgical removal of the tumour. Expression levels of the miR-500 are normalised to miR-16. N.D., not detected.

Discussion

Using a global miRNA expression profile in mouse liver development analysed by an LNA-based miRNA microarray, our data indicate that dynamic changes in miRNA expression occur in mouse liver development. However, the number of high-expressed miRNAs was quite limited at all developmental stages of the liver. This finding is also consistent with several reports that dominant miRNA expression is rigidly controlled in a developmental stage-specific and tissue- or cell-type-specific manner (Chen et al 2006, Shan et al. 2007). For example, it has been reported that the expressions of miR-1 and miR-133 are high and specific in adult cardiac and skeletal muscles and modulate skeletal muscle proliferation and differentiation by negatively regulating the histone deacetylase-4 or serum response factor (Chen et al. 2006). On the other hand, expression levels of the general miRNA are low at all stages of liver development. However, our data indicate that the expression pattern of some of the low-expressed miRNAs, including let-7 family, also dramatically change in the process of mouse liver development (Figure 4B). Using this platform, the overall regulation of individual miRNAs of sequential stages of liver development was determined, providing us with a useful baseline for understanding the developmental dynamics of liver miRNA expression.

In this study, we identified a novel cancer biomarker candidate, miR-500, which was designated as an oncofetal miRNA in the early stage of liver cancer, because miR-500 expression is highly expressed in a fetal liver and downregulated in the developmental process and then upregulated in the process of liver cirrhosis. When the expression profile of miR-500 in human tissues was examined, its expression was not specific in the liver and was broadly detected in all tissues (see Supplementary Figure 3 in the online version of this article). However, the expression level of miR-500 is high at the early stages of liver development in mice and humans. Furthermore, miR-500 was abundantly expressed in human liver cancer cell lines (JHH-7, Huh-7, HepG2, Hep3B and Alexander) and liver cancer tissues. Interestingly, six miRNAs (mir-532, 188, 362, 501, 660, 502) in addition to miR-500 make a cluster within a 10-kb distance from miR-500, and their expressions could be modulated by the same transcriptional regulatory unit. However, the levels were not remarkably changed during mouse liver development. Therefore, by analysing these miRNAs together, miR-500 might be a better biomarker in HCC.

We tried to test the effect of miR-500 using liver cancer cell lines. In a knock-down analysis of miR-500 with miR-500 LNA, significant changes in cell proliferation and colony formation were not observed in both Alexander and JHH-7 cells (see Supplementary Figure 5A and B in the online version of this article). Likewise, mature

miR-500 were transfected into Li-7 cells, which did not express miR-500 and we found there are no significant differences in cell proliferation (see Supplementary Figure 5C in the online version of this article). Although our data indicated that miR-500 did not affect cell proliferation in liver cancer cell lines, there might be a close association between tissue development and carcinogenesis in the fields of miRNA. For detailed analysis of function of miR-500, we await for generation of miR-500 knockout mice.

As several groups have reported that levels of certain circulating miRNA are associated with clinical characteristics in diseases (Gilad et al. 2008, Lawrie et al. 2008), our data suggest that miR-500 was circulating in the sera of the HCC patients and miR-500 levels in sera of the HCC patients returned to normal after the surgery. Although our results are promising for miRNA-based HCC screening, there are several limitations in this study and we suggest: (1) as the sample size is quite small, further validation that miR-500 could be a reliable marker for HCC in a large cohort is necessary; (2) use of better controls to determine whether or not serum miR-500 levels are changed due to the trauma of surgery; (3) it is desirable to examine whether serum miR-500 levels change in patients with chronic hepatitis and liver cirrhosis; (4) it is necessary to compare if serum miR-500 could be better than earlier diagnostic methods such as serum α-fetoprotein.

The differential expression patterns of miR-101b, miR-122a, miR-142-5p, miR-223 and miR-451 were determined by miRNA microarray and real-time PCR analysis. The specific expression of miR-122 in the liver has previously been described by several research groups. Esau et al. (2006) reported that miR-122 was a key regulator of lipid metabolism in the liver, regulating increased hepatic fatty acid oxidation, a decrease in hepatic fatty acid and cholesterol synthesis rates by reductions of several lipogenic genes. Interestingly, two groups demonstrated evidence that the hepatitis C virus genome has predicted binding sites of miR-122 and that miR-122 positively regulated the replication hepatitis C virus in human liver (Jopling et al. 2005, Randall et al. 2007). In addition to miR-122a, we found that miR-101b expression was upregulated in mouse liver development. Furthermore, upregulation of miR-101b and miR-122a expression was observed in the *in vitro* cultured of fetal hepatocytes treated with OsM and Dex (see Supplementary Figure 4A–C in the online version of this article). It has been reported that miR-101 is related to the immune system and megakaryocytopoiesis (Yu D et al. 2007, Garzon et al. 2006); however, the role of miR-101 in the liver has not yet been examined.

During early development in mice, haematopoietic stem cells emerge in the aorta/gonado/mesonephros region and then the stem cells migrate and expand in the fetal liver before haematopoiesis takes place in the bone marrow by the time of birth. Although most of the miRNAs that we observe in the liver developmental process are constitutively expressed, specific miRNAs are enriched at distinct stages of haematopoietic development. We found that the expression of miR-142-5p, miR-223 and miR-451 was downregulated in the process of liver maturation. As it has been reported that miR-142-5p and miR-142-3p are highly expressed in all haematopoietic tissues (Chen et al. 2004), miR-142 may thus play a critical role at the early stage of haematopoiesis. The expression of miR-223 was mainly detected in bone marrow and negatively regulated myeloid progenitor proliferation and granulocytic differentiation and activation (Johnnidis et al. 2008). In addition, miR-451 expression was upregulated during erythroid differentiation, and gain- and loss-of-function studies disclosed that miR-451 was related to erythroid maturation (Zhan et al. 2007).

Recent studies have indicated that a decrease of mature miRNA expression by impaired miRNA processing accelerates tumorigenesis and that a global reduction of miRNAs is observed in human cancers, suggesting that the role of overall miRNAs is to guard against oncogenic transformation (Kumar et al. 2007, Lu et al. 2005). In particular, the let-7 family is broadly known as a tumour suppressor. It has been reported that a decrease of let-7 expression was observed in human lung cancer and that let-7 negatively regulates the expression of H-ras and *HMGA2* oncogenes in breast cancer cells (Johnson et al. 2005, Yu F et al. 2007, Takamizawa et al. 2004). In addition, miR-16 was also reported as a tumour suppressor by inducing apoptosis mediated by Bcl-2 and modulating the cell cycle (Cimmino et al. 2005, Linsley et al. 2007). In a study of liver carcinogenesis, a decrease of miR-122 expression was observed in rat liver tumour (Kutay et al. 2006). Consistent with this report, miR-122a and miR-101b expression levels in 40 pairs of malignant neoplasias of hepatocyte lineage and adjacent non-tumorous tissue were reduced significantly ($p<0.05$, $n=40$) in tumour samples (see Supplementary Figure 4D in the online version of this article). However, in previous studies, it has been revealed that specific miRNAs acted as oncogenes, as their overexpression facilitates cancer progression. For example, miR-17-92 polycistron was overexpressed in lymphomas, lung cancers and colorectal cancers and enhanced cell proliferation (He et al. 2005, Hayashita et al. 2005). Furthermore, the copy number and expression level of miR-155 and its non-coding RNA transcript BIC were greatly increased in B-cell lymphomas (Eis et al. 2005). Our data show that the expression profile of oncogenic miRNAs was downregulated and, vice versa, the expression of tumour-suppressor miRNAs was upregulated in the process of liver development (Figure 4). This suggests that elevated oncogenic miRNAs are important

at the early developmental stage of the liver because, in this period, cell proliferation is frequent; in contrast, upregulation of tumour suppressor miRNAs is essential for preventing abnormal cell proliferation at the late stage of liver development. Therefore, our data suggest that the tight regulation of expression of cancer-related miRNAs (both oncogenic miRNAs and tumour suppressor miRNAs) occurred during normal liver development.

Finally, we have documented dynamic changes in miRNA expression that were found in the process of mouse liver development and some of them behaved as an oncofetal miRNA in HCC. Although little is known about the expression regulations, targets or roles of miRNAs in the liver, the expression profiles of miRNA in development could be informative with respect to the elucidation of the process of the development and diagnosis of cancer because the expression of some of the cancer-related miRNAs dramatically changed. Further studies on the differential expression of miRNA in liver development could contribute to a better understanding of the process of liver development and embryonic haematopoiesis and could facilitate the discovery of candidate miRNAs for cancer diagnosis and therapeutic targets in liver cancer.

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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

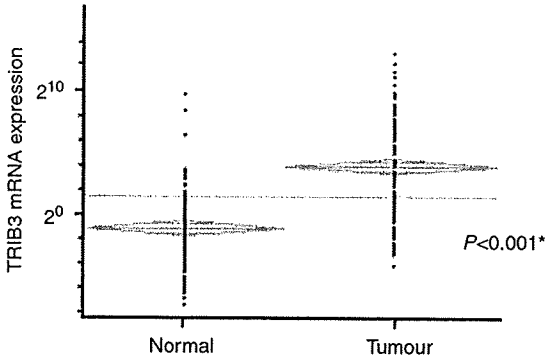

Figure 1 *TRIB3* mRNA expression in clinical tissue specimens. Quantitative real-time RT–PCR on 202 paired clinical samples showed that 181 of 202 (89.6%) samples had higher levels of *TRIB3* mRNA in tumours than in paired normal regions. The mean expression value of *TRIB3* mRNA in tumour regions, 154.62 ± 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.

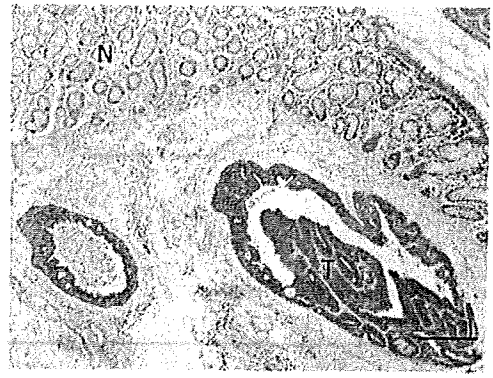

Figure 2 Immunohistochemical staining for Trib3 in tumour and normal specimens. A representative positive stain for Trib3 in tissues from CRC patients. Positive staining is observed in the nucleus and cytoplasm of cancer cells, but not in stromal cells. Trib3 expression was associated with mRNA expression. 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.

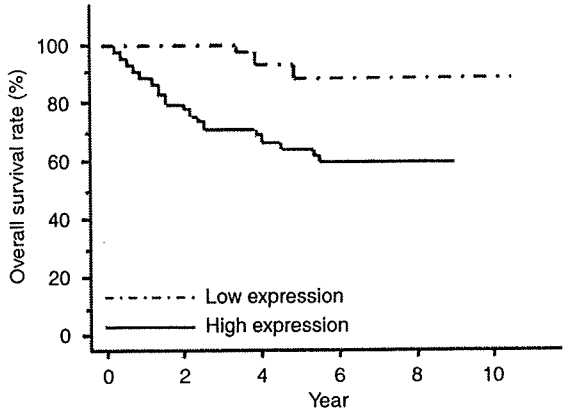

Figure 3 Overall survival rates of patients with CRC on the basis of TRIB3 mRNA expression status. The overall survival rate was significantly lower in the *TRIB3* high-expression group than that in the low-expression group ($P<0.001$). CRC = colorectal cancer; TRIB3 = tribbles homologue 3.

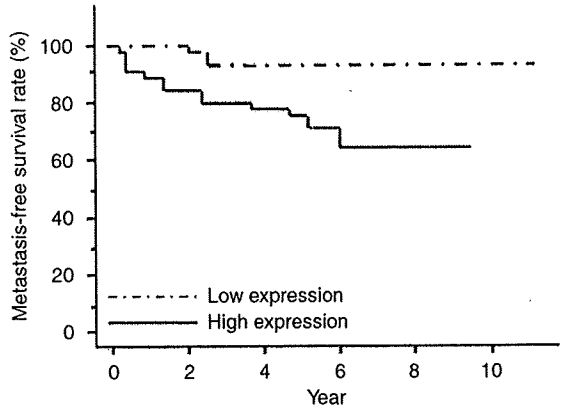

Figure 4 Metachronous metastasis-free over 5 years' survival rates of patients with CRC in stages I II, and III, on the basis of TRIB3 mRNA expression status. The metachronous metastasis-free over 5 years' survival rate was significantly lower in patients with the *TRIB3* high-expression group compared 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

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 *TRIB3* 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.

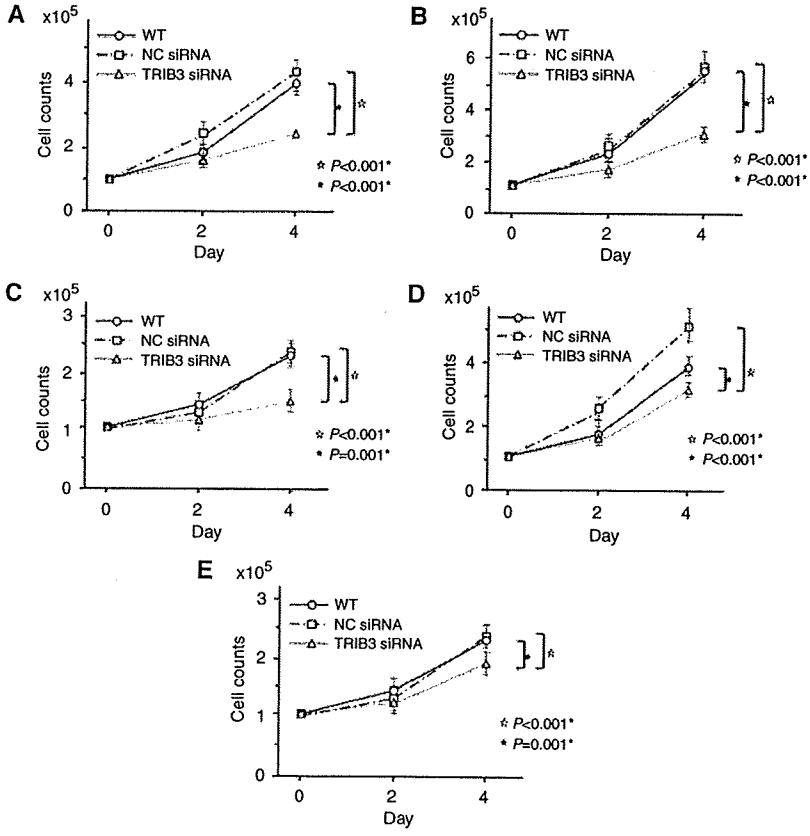

Figure 5 Proliferation assay with siRNA inhibition in five CRC cell lines. Proliferation assay was performed in five CRC cell lines (**A**, DLD-1; **B**, LoVo; **C**, HCT-116; **D**, KM12SM; E, SW480). There were significant differences between WT or NC, and *TRIB3* siRNA. Values are presented means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

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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Bone marrow and peripheral blood expression of *ID1* in human gastric carcinoma patients is a *bona fide* indicator of lymph node and peritoneal metastasis

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Recent studies have showed that the bone marrow-derived endothelial progenitor cells play critical roles in metastasis and that *ID1* is required in metastasis as regulator of angiogenesis. Therefore, we investigated the clinical significance of *ID1* mRNA expression in bone marrow and peripheral samples in patients with gastric cancer. Two hundred and eighty-nine bone marrow and 196 peripheral blood samples from gastric cancer patients were collected and analysed by quantitative RT–PCR for *ID1*. The ID1 protein expression in one bone marrow, three metastatic lymph nodes and three peritoneal disseminated tumours was examined by immunohistochemical methods. In both bone marrow and peripheral blood samples, *ID1* mRNA expression in the metastatic group was significantly higher than in any other group ($P=0.003$, $P=0.0001$, respectively) and significantly associated with lymph node metastasis and peritoneal dissemination. The cells in bone marrow with metastatic cancer stained strongly with ID1 compared with those of healthy volunteers. The expression of *ID1* mRNA in bone marrow and peripheral blood was significantly associated with lymph node metastasis and peritoneal dissemination, and therefore constitutes a predictable marker for lymph node metastasis and peritoneal dissemination.
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Keywords: *ID1*; gastric cancer; bone marrow; peripheral blood; lymph node metastasis; peritoneal dissemination

The presence of isolated tumour cells (ITCs) is an important factor in the metastasis of solid cancers. Recently, we investigated the presence of ITCs in peripheral blood and in bone marrow, using quantitative RT–PCR in more than 800 cases of gastric cancer (Mimori *et al*, 2008). We found that ITCs circulate in a range of clinical stages of gastric cancer. These data suggested that host cells might play a supportive role for metastasis.

Recently, Kaplan *et al* reported that bone marrow-derived haematopoietic progenitor cells that express vascular endothelial growth factor receptor 1 (VEGFR-1) home to tumour-specific pre-metastatic sites and form cellular clusters before the arrival of tumour cells (Kaplan *et al*, 2005). In a large-scale study of gastric cancer cases, we recently reported that the simultaneous expression of ITC-associated genes and high levels of expression of *VEGFR-1* in bone marrow were significantly associated with haematogeneous metastases (Mimori *et al*, 2008). Gao *et al* determined that the bone marrow-derived endothelial progenitor cells (EPCs) were critical regulators of angiogenic switching (Gao *et al*, 2008). Furthermore, they showed that tumours induce expression of *ID1* in EPCs and that suppression of *ID1* after metastatic colonisation blocked EPC mobilisation, inhibited angiogenesis and impaired pulmonary macrometastases. ID proteins are inhibitors of DNA binding of basic helix-loop-helix (bHLH) transcription factors by heterodimerisation with the bHLH proteins (Benezra *et al*, 1990). *ID1* has been reported to be associated with the undifferentiation of cancer cells, severe malignant grade of tumour, invasion of tumours and worse prognosis in several tumours (Fong *et al*, 2003).

In this study, we investigated the clinical significance of the *ID1* mRNA expression in bone marrow and peripheral blood samples obtained from gastric cancer patients. The results showed that the *ID1* mRNA expression in bone marrow and peripheral blood was significantly associated with lymph node metastasis and peritoneal dissemination. Thus, *ID1* is a *bona fide* predictive marker for both pathologic parameters, each of which is an established definitive prognostic indicator in gastric cancer.

MATERIALS AND METHODS

Patients

Physicians (TF and MS) collected bone marrow and peripheral blood samples from 289 Japanese gastric cancer patients who underwent surgery from 2001 to 2004 at the Central Hospital, the

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Table 1 Clinicopathlogial significance of *ID1* mRNA expression in gastric cancer patients

Features	Bone marrow			Peripheral blood		
	Number	*ID1* mRNA expression (mean ± s.d.)	*P*-value	Number	*ID1* mRNA expression (mean ± s.d.)	*P*-value
Age	289	62.3 ± 11.9	—	196	62.9 ± 12.2	—
Sex (M:F)	289	190:99	—	196	131:65	—
Tumour size			0.01			0.002
⩽5 cm	126	311 ± 146		85	24.8 ± 12.2	
⩾5 cm	160	795 ± 129		110	64.6 ± 10.7	
Depth of tumour invasion[a]			0.01			0.004
m, sm	90	210 ± 171		62	12.9 ± 14.2	
mp, ss, se, si	199	744 ± 115		134	62.8 ± 9.6	
Venous invasion			0.97			0.007
Positive	72	580 ± 199		50	77.8 ± 15.2	
Negative	188	571 ± 123		128	28.9 ± 9.5	
Lymphatic invasion			0.001			0.02
Positive	156	850 ± 132		110	57.8 ± 10.3	
Negative	104	158 ± 162		68	18.2 ± 13.1	
Lymph node metastasis			0.001			0.02
Positive	188	800 ± 121		132	56.7 ± 9.6	
Negative	79	58.7 ± 186		50	12.4 ± 15.6	
Peritoneal dissemination[b]			0.002			<0.0001
Positive	70	1102 ± 194		40	119 ± 17.1	
Negative	218	412 ± 110		156	28.6 ± 8.6	

[a]Tumour invasion of mucosa (m), submucosa (sm), muscularis propria (mp), subserosa (ss), penetration of serosa (se) and invasion of adjacent strucures (si). [b]Peritoneal dissemination: peritoneal cytology or metastasis positive.

National Cancer Center, Tokyo, Japan. The documented informed consent was obtained from all patients and the protocol of the study was approved by the local ethics committee. There were 190 male and 99 female patients with an average age of 62.3 and a range of 24–86 years (Table 1). Seventy of the patients showed peritoneal dissemination at the time of surgery or at postoperative follow-up. Among the 289 cases, 76, 60, 62 and 91 were classified as stages I, II, III or IV, respectively, according to the Treaty for Japanese Gastric Cancer Association (Maruyama *et al*, 2006).

Bone marrow and peripheral blood samples from gastric cancer patients

Aspiration of both bone marrow and peripheral blood was conducted under general anaesthesia immediately before surgery as described earlier (Mimori *et al*, 2008). The bone marrow aspirate was obtained from the sternum using a bone marrow aspiration needle and peripheral blood was obtained through a venous catheter. The first 1.0 ml of bone marrow and peripheral blood were discarded to avoid contamination by the skin. The second collected 1.0 ml of bone marrow and peripheral blood were put into 4.0 ml of Isogen-LS (Nippon Gene, Toyama, Japan) and stored at −80 °C until RNA extraction.

Total RNA extraction and first-strand cDNA synthesis

Samples transferred from Tokyo to Beppu remained frozen while in transit. Total RNA was extracted from bone marrow and peripheral blood according to the manufacturer's protocol as described elsewhere (Iinuma *et al*, 2006). The reverse transcriptase reaction (RT) was performed as described earlier (Mori *et al*, 1995). The first-strand cDNA was synthesised from 2.7 μg of total RNA in 30 μl reaction mixtures containing 5 μl 5 × RT buffer (BRL, Gaithersburg, MD, USA), 200 μM dNTP, a 100 μM solution of a random hexadeoxynucleotide mixture, 50 units of Rnasin (Promega, Madison, WI, USA), 2 μl of 0.1 M dithiothreitol and 100 units of Maloney leukemia virus RT (BRL). The mixture was incubated at 37°C for 60 min, heated to 95°C for 10 min and then chilled on ice.

Quantitative real-time RT–PCR

The sequences of *ID1* mRNA were as follows: sense, 5′-CC AGTGGCAGCACCGCCACC-3′, and anti-sense, 5′-CGGATTCCG AGTTCAGCTCC-3′. We used glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*) as an internal control. The primers were as follows: sense, 5′-TTGGTATCGTGGAAGGACTCTA-3′, and anti-sense, 5′-TGTCATATTTGGCAGGTT-3′. Real-time monitoring of PCR reactions was performed using the LightCycler system (Roche Applied Science, Indianapolis, IN, USA) and SYBER-Green I dye (Roche Diagnostics, Tokyo, Japan) to detect *ID1* in bone marrow and peripheral blood. Monitoring was performed according to the manufacturer's instructions, as described earlier (Ogawa *et al*, 2005). In brief, a master mixture was prepared on ice, containing 1 μl of cDNA, 2 μl of DNA Master SYBER-Green I mix, 50 ng of primers and 2.4 μl of 25 mM $MgCl_2$. The final volume was adjusted to 20 μl with water. After the reaction mixture was loaded into glass capillary tubes, quantitative RT–PCR was performed with the following cycling conditions: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, annealing at 62°C for 10 s and extension at 72°C for 10 s. After amplification, products were subjected to a temperature gradient from 67°C to 95°C at 0.2°C s^{-1}, under continuous fluorescence monitoring, to produce a melting curve of products.