British Journal of Cancer (2012) 107, 1345 – 1353

www.bjcancer.com

Clinical significance of *miR-144-ZFX* axis in disseminated tumour cells in bone marrow in gastric cancer cases

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BACKGROUND: We previously reported that bone marrow (BM) was a homing site for gastric cancer (GC) cells leading to haematogenous metastases. There has been little study that microRNAs regulated pathways in malignant cells or host cells in BM, and thereby regulated the progression of GC.

METHODS: Both microRNA microarray and gene expression microarray analyses of total RNA from BM were conducted, comparing five early and five advanced GC patients. We focused on *miR-144-ZFX* axis as a candidate BM regulator of GC progression and validated the origin of the microRNA expression in diverse cell fractions ($EpCAM^+CD45^-$, $EpCAM^-CD45^+$, and $CD14^+$) by magnetic-activated cell sorting (MACS).

RESULTS: Quantitative reverse-transcriptase (RT)–PCR analysis validated diminished *miR-144* expression in stage IV GC patients with respect to stage I GC patients (*t*-test, $P = 0.02$), with an inverse correlation to *ZFX* (ANOVA, $P < 0.01$). Luciferase reporter assays in five GC cell lines indicated their direct binding and validated by western blotting. Pre-*miR144* treatment and the resultant repression of *ZFX* in GC cell lines moderately upregulated their susceptibility to 5-fluorouracil chemotherapy. In MACS-purified BM fractions, the level of *miR-144* expression was significantly diminished in disseminated tumour cell fraction ($P = 0.0005$). Diminished *miR-144* expression in 93 cases of primary GC indicated poor prognosis.

CONCLUSION: We speculate that disseminated cancer cells could survive in BM when low expression of *miR-144* permits upregulation of *ZFX*. The regulation of the *miR-144-ZFX* axis in cancer cells has a key role in the indicator of the progression of GC cases.

British Journal of Cancer (2012) **107,** 1345–1353. doi:10.1038/bjc.2012.326 www.bjcancer.com
Published online 6 September 2012

Keywords: cell fraction; microRNA; disseminated tumour cell; host cells

Cancer initiation and progression may involve microRNAs, which are small non-coding RNAs that can regulate gene expression (Calin and Croce, 2006b). Recent studies of microRNA expression profiles of primary human tumours have identified expression signatures associated with classification, diagnosis, prognosis, and even outcome after treatment (Calin *et al*, 2004; Iorio *et al*, 2005; Yanaihara *et al*, 2006; Bottoni *et al*, 2007; Calin and Croce, 2007; Gottardo *et al*, 2007; Kulshreshtha *et al*, 2007; Visone *et al*, 2007; Garzon *et al*, 2008; Liu *et al*, 2008a, b; Lujambio *et al*, 2008; Marcucci *et al*, 2008; Schetter *et al*, 2008; Zhang *et al*, 2008). Profiling has also been used to identify microRNAs that might represent downstream targets of activated oncogenic pathways in primary cancers (Calin and Croce, 2006a). However, there have been few efforts in gastric cancer (GC) to identify bone marrow (BM) microRNAs and the target genes involved in the progression of disease. The primary endpoints of such studies are the establishment of significant microRNA/target gene profiles in BM and a determination of how they contribute to the progression of GC. This information could clarify how a given microRNA gene axis in BM cells contributes to the progression of GC.

Primary cancer cells are released from their initial sites and spread via the peripheral circulation to the BM. However, both circulating tumour cells and host factors in BM can determine whether metastasis and/or recurrence is likely after curative surgery. It is believed that few cancer stem cells (CSC) initially have the capacity for tumorigenesis and cancer metastasis (Alix-Panabieres *et al*, 2008; Dirks, 2010). Kang (2009) disclosed the metastatic behaviour of CSCs derived from tumours. As a result, we have focused our studies on CSCs in BM. Kaplan *et al* (2005) reported the important role of BM progenitor cells as niches for tumour cells (Lyden *et al*, 2001). After receiving signals from primary GC cells, haematopoietic progenitor cells from BM or endothelial progenitor cells in peripheral blood are recruited to pre-metastatic niches (Mimori *et al*, 2008). Thus, primary circulating tumour cells actively move to pre-metastatic niches, leading to the development of metastasis. It is postulated that both host BM cells and circulating tumour cells have important roles in the progression of malignant disease. However, it is not known which cells in BM are responsible for the progression of GC in a

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Received 22 December 2011; revised 28 May 2012; accepted 31 May 2012; published online 6 September 2012

supportive niche. To approach this problem, we identified the microRNAs expressed among diverse BM cell populations, including the disseminated tumour cell fraction.

Using two microarray platforms, we initially focused on candidate microRNAs regulating GC progression. We postulated that the use of two independent microarray analyses in a double-blind manner would ensure reliability of the data. Then, candidate target genes were subjected to further analysis using several mammalian microRNA target databases, such as miRanda, TargetScan, and PICTAR. We performed gene expression microarray analysis of BM from GC cases that were applied to the microRNA microarray study. We found genes overexpressed in BM in stage IV GC patients. On the basis of *in silico* analyses, we then compared the genes expressed in BM in stage IV GC patients to determine their contribution to progression. Quantitative RT–PCR assays and assessment of direct binding by luciferase assays were conducted to validate the clinical significance of microRNA-target gene expression.

PATIENTS AND METHODS

Patients

We performed a microRNA microarray comparison between microRNA isolated from the BM of five GC cases with peritoneal dissemination and five cancer cases in whom invasion did not reach the sub-mucosal layer, and lymph node metastasis was absent in our hospital (Supplementary Table S1).

Then, physicians collected BM samples from 205 Japanese GC patients who underwent surgery from 2001 to 2004 at the Central Hospital, National Cancer Center, Tokyo, Japan. Documented informed consent was obtained from all patients, and the local ethics committee approved the study protocol. The average age of the 131 male and 74 female patients was 61.5 years, with a range of 27–86 years. On the basis of the Treaty for Japanese Gastric Cancer Association (Maruyama *et al*, 2006), 52 cases were classified as stage I, 49 cases as stage II, 51 cases as stage III, and 53 cases as stage IV. Normal negative controls consisted of BM samples collected from 20 patients with no malignancies (e.g., gallstone and hernia cases) from April 2000 to March 2003. Among the 205 cases, we randomly selected 10 representative GC cases, 5 in stage IV and 5 in stage I for the two microarray studies.

Total RNA from 93 cases of primary GCs in our hospital were collected and examined for further analysis.

BM collection

Aspiration of BM was conducted under general anaesthesia immediately before surgery as previously described (Mimori *et al*, 2008). The BM aspirate was obtained from the sternum using a BM aspiration needle. A volume of 3 ml of BM was added to 4.0 ml of Isogen-LS (Nippon Gene, Toyama, Japan), which was shaken vigorously and stored at $-80\,^{\circ}C$ until RNA extraction.

Gene expression profiles in stage IV and stage I GC cases: comparison with database of predicted target genes

We performed microarray analyses of BM-extracted RNA in five cases of stage IV and those of stage I using Toray's platform (Nagino *et al*, 2006; Ito *et al*, 2007; Iwahashi *et al*, 2007). It is possible to monitor gene expression profiles even with very small amounts of RNA (0.1–0.01 μg of total RNA) without amplification. Using the 3D-gene platform technology, RNA samples can be detected down to the attomole level.

Total RNA extraction and first-strand complementary DNA synthesis

Samples transferred from Tokyo to Beppu remained frozen while in transit. Total RNA was extracted from BM according to the manufacturer's protocol as described elsewhere (Liu *et al*, 2008a). The RT reaction was performed as previously described (Mori *et al*, 1995). The first complementary DNA (cDNA) strand was synthesised from 2.7 μg of total RNA in 30 μl reaction mixtures containing 5 μl 5 $\times$ RT buffer (Gibco BRL, Gaithersburg, MD, USA), 200 μM deoxynucleotide triphosphates (dNTPs), 100 μM 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 leukaemia virus RT (BRL, Tokyo, Japan). The mixture was incubated at $37\,^{\circ}C$ for 60 min, heated to $95\,^{\circ}C$ for 10 min, and then chilled on ice.

microRNA expression profiling by two independent experiments: the first assay

Extracted total RNA was labelled with Hy5 using the miRCURY LNA Array miR labelling kit (Exiqon, Vedbaek, Denmark). Labelled RNAs were hybridised onto 3D-Gene Human microRNA Oligo chips containing 837 anti-sense probes printed in duplicate spots (Toray, Kamakura, Japan). The annotation and oligonucleotide sequences of the probes conformed to the miRBase microRNA database (http://microRNA.sanger.ac.uk/sequences/). After stringent washes, fluorescent signals were scanned with the ScanArray Express Scanner (PerkinElmer, Waltham, MA, USA) and analysed using GenePix Pro version 5.0 (Molecular Devices, Sunnyvale, CA, USA). The raw data for each spot was normalised by substitution with a mean intensity of the background signal determined from all blank spots' signal intensities with 95% confidence intervals. Measurements of duplicate spots with signal intensities greater than two s.d. of the background signal intensity were considered to be valid. The relative expression level of a given microRNA was calculated by comparing the signal intensities of the averaged valid spots with their mean value throughout the microarray experiments after normalisation by their median values. MicroRNAs differentially expressed between GC stage I and IV were statistically identified using the Welch *t*-test.

microRNA microarray analysis: the second assay

The concentrations and purities of total RNAs were assessed spectrophotometrically, and RNA integrity was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Tokyo, Japan). The RNA labelling and hybridisation on microRNA microarray chips were performed as described elsewhere (Liu *et al*, 2008a). Briefly, 5.0 μg of total RNA from each sample were reverse transcribed using a biotin end-labelled random-octamer oligonucleotide primer. Hybridisation of biotin-labelled cDNA was performed on Ohio State University's microRNA microarray chip (version 4.0, microRNACHIPv4), which contains 4104 microRNA probes, including 474 human and 373 mouse microRNA genes, spotted in duplicate. The hybridised chips were washed and processed to detect biotin-containing transcripts by streptavidin–Alexa647 conjugate and scanned on an Axon 4000B microarray scanner (Molecular Devices).

Quantitative real-time RT–PCR for microRNA

We performed quantitative analysis of *miR-144*. We synthesised *miR-144*- and RNU6B (internal control)-specific cDNAs from total RNA using gene-specific primers according to the TaqMan MicroRNA Assays Protocol (Roche Applied Science, Indianapolis, IN, USA). Reverse transcriptase reactions contained 10 ng total RNAs, 50 nmol l^{-1} stem-loop RT primer, 1 $\times$ RT buffer, 0.25 mmol l^{-1} each dNTP, 3.33 units per μl MultiScribe RT, and 0.25 units per μl RNase inhibitor. Reactions were incubated in a BIO-RAD i-Cycler (Bio-Rad Laboratories, Tokyo, Japan) in 96-well plates for 30 min at $16\,^{\circ}C$, 30 min at $42\,^{\circ}C$, 5 min at $85\,^{\circ}C$, and then maintained at $4\,^{\circ}C$. Real-time PCR was done using an Applied

Biosystems 7500 real-time PCR system. The 10 μl PCR mixture included 0.67 μl RT products, 1 × TaqMan Universal PCR master mix, and 1 μl primers and TaqMan microRNA Assays probe mix. Reactions were incubated in 96-well optical plates at 95 °C for 10 min. Relative quantification of microRNA expression was calculated as follows: raw data were presented as the relative quantity of target microRNA normalised with respect to RNU6B and relative to a calibrated sample.

Quantitative RT–PCR for candidate genes

The following primer sequences were used to amplify *ZFX* mRNA (Sakhinia *et al*, 2007); *ZFX* forward: 5′-ATA GCA CTA CAG ATG CCT CAG-3′, and *ZFX* reverse: 5′-GAA TGA GAC TGA TTG GCT TTA-3′. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control, with the following primers: sense, 5′-TGA ACG GGA AGC TCA CTG G-3′; antisense, 5′-TCC ACC ACC CTG TTG CTG TA-3′. Real-time monitoring of PCR reactions was performed using the LightCycler System (Roche Applied Science) and SYBR-Green I dye (Roche Diagnostics, Tokyo, Japan). Monitoring was performed according to the manufacturer's instructions, as previously described (Mimori *et al*, 2008). In brief, reactions were prepared on ice, containing 1 μl of cDNA, 1 × DNA Master SYBR-Green I, 50 ng of primers, and 3 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, the amplicons 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 the products.

Data analysis for RT–PCR

After proportional baseline adjustment, the fit point method was employed to determine the cycle in which the log-linear signal was first distinguishable from the baseline. This cycle number was used as the crossing point value. Measuring the crossing point of each standard value and plotting it against the logarithmic concentration value produced a standard curve. Concentrations of unknown samples were calculated by plotting their crossing points against the standard curve and dividing by the *GAPDH* content.

After raw data were measured, we subtracted the background value, which yielded the background value (average) + 2 s.d. (BG substitution). The value was converted into $\log_2$ data (BG substitution (LOG_2)). Then, we found the z-transform value as the standard normal distribution value adjusted by the normal distribution.

Western blot analysis

Total protein was extracted from cell lines using protein extraction solution (PRO-PREP, iNtRON Biotechnology, Seongnam Si, Korea). Total protein (40 μg) was electrophoresed in 10% concentration READY GELS J (Bio-Rad Laboratories) and electroblotted onto pure nitrocellulose membranes (Trans-Blot Transfer Medium; Bio-Rad Laboratories) at 0.2 A for 120 min. The ZFX protein was detected using rabbit polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1 : 200. The ZFX protein level was normalised to the level of β-actin protein (Cytoskeleton, Denver, CO, USA) diluted 1 : 1000. Blots were developed with horseradish-peroxidase-linked anti- rabbit immunoglobulin (Promega) diluted 1 : 2000. The ECL Detection Reagents (Amersham Biosciences, Piscataway, NJ, USA) were used to detect antigen–antibody reactions.

Cell culture and luciferase assay

Note also that the detection and characterisation of micrometastatic cells or host cells in the BM of patients with cancer are of prognostic and therapeutic importance (Braun *et al*, 2000, 2005; Choesmel *et al*, 2004). Therefore, we examined and compared levels of microRNA expression in cellular fractions, such as epithelial cancer cells ($EpCAM^+CD45^-$), haematopoietic cells ($EpCAM^-CD45^+$), macrophages ($CD14^+$), and other fractions separated by magnetic-activated cell sorting (MACS; Schmitz *et al*, 1994; Engel *et al*, 1999). On the basis of the analysis of cell populations, we identified the origin of cells expressing specific microRNA-target gene pathways in the BM.

The 3′-UTR and ORF of *ZFX* was amplified by RT–PCR. The amplified product was subcloned and ligated into the pmirGLO Dual-Luciferase microRNA Target Expression Vector (Promega). The resultant reporter vector position was confirmed by sequencing and termed pmirGLO-*ZFX*. Luciferase assays were conducted using 1×10^4 MKN45 cells, MKN7 cells, AZ521 cells, MKN74 cells, and NUGC4 cells plated in a 96-well plate. Transfections were performed using Lipofectamine 2000 (Invitrogen, Tokyo, Japan) in OptiMEM-reduced serum media (GIBCO, Tokyo, Japan). Cells were transfected with 30 ng of pmirGLO-*ZFX* or pmirGLO empty vector, and either 100 nM of pre-miR-negative control or pre-*miR-144*. Twenty-four hours following transfection, cells were assayed for both firefly and Renilla luciferase using Dual-Glo Luciferase Assay System (Promega). All transfection experiments were conducted in triplicate.

Cell sorting with antibodies by MACS

We performed immuno-magnetic cell sorting of 30 ml of BM from nine GC patients (there were six cases of stage I GC and three cases of stage IV GC). The mononuclear cell fraction was isolated by carefully loading 30 ml of whole blood onto 10 ml of Ficoll density medium (GE Healthcare Bio-Sciences, Tokyo, Japan) in 50 ml polypropylene tubes. The gradients were centrifuged for 30 min at room temperature at 450 **g**, and the interface collected after aspirating and discarding the supernatant. The cells were washed with 20 ml PBS and centrifuged at 150 **g** for 5 min at room temperature. The supernatant was aspirated and the cells were washed with PBS a second time.

CD326 (EpCAM)-PE, human MicroBeads was used for the positive selection of viable epithelial tumour cells from peripheral blood. To detect human leukocytes, we used human CD45-FITC. Then, CD14-APC was applied for detection of human monocytes and macrophages (Miltenyi Biotec, Bergisch Gladbach, Germany).

We measured *miR-144* expression level per one cell to compare the average between stage I and stage IV in each fraction.

Cell culture assays for altered sensitivity to chemotherapy by anti-microRNA

To determine the role of *ZFX* as a regulator of chemotherapy sensitivity in cancer cells, we introduced pre-*miR-144*, control anti-miR, anti-*miR-144* (100 pmol), and anti-*miR-144* (200 pmol) into MKN45 parental cells. The cells were treated with 5-fluorouracil (5-FU; 0, 1, 5 and 10 μg ml^{-1}) to compare levels of toxicity.

Statistical analysis

For continuous variables, data were expressed as the means ± s.d. The relationship between *ZFX* mRNA expression and clinicopathological factors was analysed using a χ^2-test and Student's *t*-test. Findings were considered significant when the *P*-value was <0.05. All tests were performed using JMP software (SAS Institute Inc., Cary, NC, USA).

The best false discovery rate (FDR) value: (predictive pseudo positive number)/(number of genes satisfied with the threshold) = (number of genes to calculate) × (*P*-value of the gene)/number of genes below *P*-value.

RESULTS

Expression profile of microRNA microarray

We performed microRNA microarray analyses to establish microRNA expression profiles for BM cells obtained from GC patients with and without metastasis. To maximise reliability, two different platforms were applied independently (Supplementary Figure S1). We observed downregulated and upregulated microRNAs in BMs of stage IV *vs* stage I GC cases. The number of significantly downregulated microRNAs in stage IV than stage I in the Japanese platform was 29 microRNAs (FDR < 0.05; Supplementary Table S2), whereas the platform from Ohio State University revealed 13 microRNA (FDR < 0.25; Supplementary Table S3). From these results, we selected three downregulated microRNAs identified by both platforms: hsa-*miR-144*, hsa-*miR-19a*, and hsa-*miR-370*.

We also performed gene expression microarray analysis of total BM RNAs from GC patients with and without metastases. We identified 180 BM probes with more abundant expression in stage IV than stage I GC (Supplementary Table S4). Using the miRanda, TargetScan, and PICTAR databases, we identified the *ZFX* gene as a target molecule, which has a high homology to SEED sequences in the 3′-UTR region of 7mer-m8, that is, *miR-144*.

Expression profiles of genes and identification of an *miR* gene axis by real microarray analysis and three databases

We compared total RNAs from the BMs of 10 GC patients, 5 cases in stage I and 5 cases in stage IV. Supplementary Table S4 shows the 180 upregulated genes in the BMs of stage IV GC cases *vs* stage I. The *ZFX* gene was significantly overexpressed in BM from stage IV GC cases with respect to stage I ($P = 0.00006$; Supplementary Table S4).

Candidate target genes for *miR-144* were predicted by three databases (miRands, Target Scan, and Pictar). We also undertook quantitative comparisons of gene expression in patients with stage IV and stage I GC. Five genes (*ZFX*, *FOSB*, *SUCLA2*, *LSM14A*, and *HDHD2)* were expressed at significantly higher levels in stage IV than stage I ($P < 0.05$). These genes are candidate *miR-144* target genes according to the three databases (Supplementary Table S5 and Supplementary Figure S1). Among the five genes, we focused on *ZFX* as a possible candidate target gene for *miR-144*.

ZFX is an X-linked, zinc finger protein. Galan-Caridad *et al* (2007) reported that the deletion of Zfx impaired the self-renewal

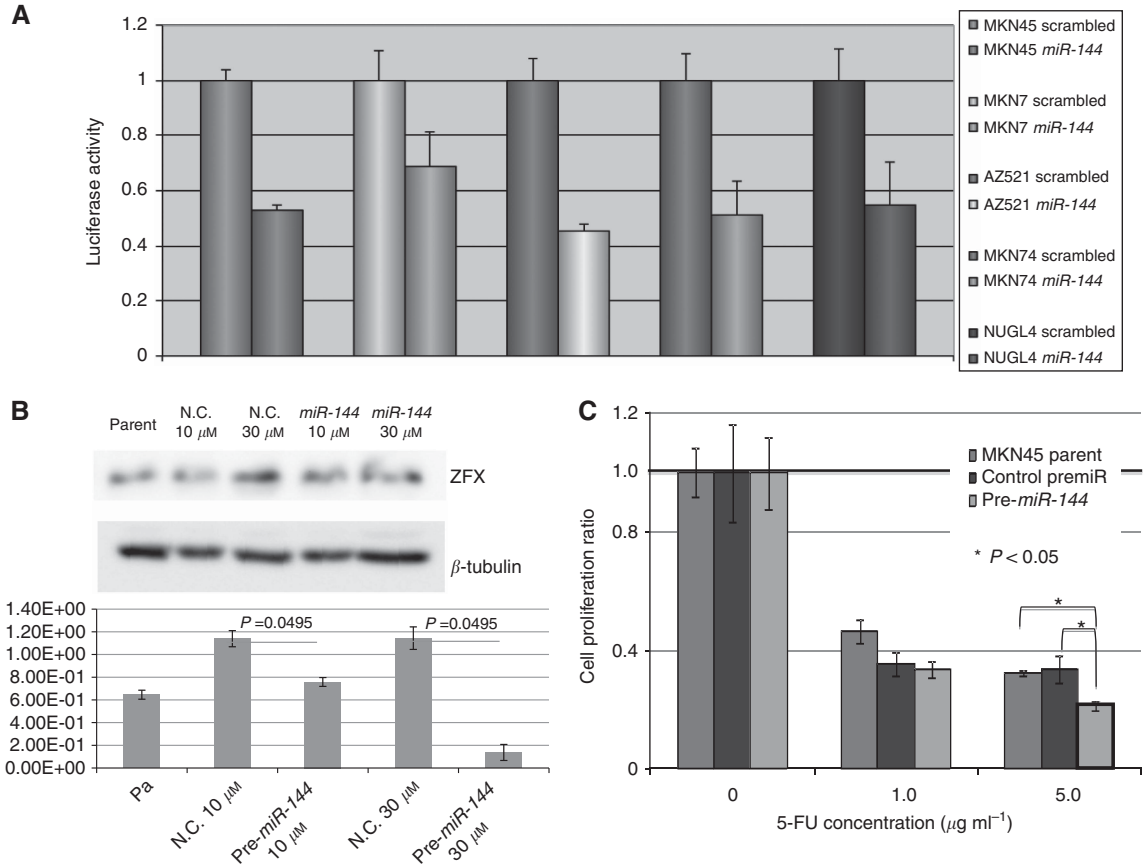

Figure 1 (**A**) Luciferase assay for interaction of *miR-144* and *ZFX* gene in five GC cell lines, MKN45, MKN7, AZ521, MKN74, and NUGC4. Every cell line attenuated with *miR-144* showed reduced luciferase activity in comparison with control cells. (**B**) Western blotting assay for disclosing the inhibitory effect of *miR-144* on ZFX protein expression. The average ZFX expression in parent cells, normal control cells with 10 μM of control vector, pre-*miR144* 10 μM, normal control cells with 30 μM of control vector, and pre-*miR144* 30 μM were 6.46E-01, 1.14E + 00, 7.58E-01, 1.14E + 00, and 1.37E-01, respectively. There were significant differences between pre-miR and control vector in each concentration. (**C**) Pre-*miR144* administration acquired the capability of resistance to 5-FU treatment. Anti-tumour effects of 5-FU (0, 1.0, and 10.0 μg ml^{-1}) treatment evaluated by cell proliferation (MTT) assay were reduced 0.94, 0.85, and 0.76 times in the group of pre-*miR144* administration in comparison with control cell lines with statistical significance by ANOVA analysis ($P < 0.05$).

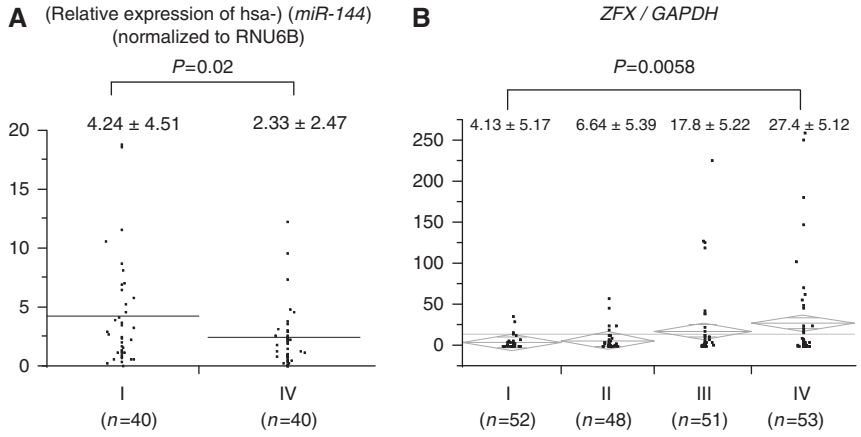

Figure 2 Verification of the expression of *miR-144* and *ZFX* in BM from 80 and 214 GC patients, respectively. (**A**) *miR-144* expression was diminished in BM from stage IV GC patients (2.33 ± 2.47) compared with those of stage I (4.24 ± 4.51; $P<0.02$). (**B**) As expected, *ZFX* expression increased with Dukes stage: 4.13 ± 5.17 (stage I), 6.64 ± 5.39 (stage II), 17.8 ± 5.22 (stage III), and 27.4 ± 5.12 (stage IV) ($P<0.0058$).

of murine embryonic stem cells. Furthermore, Zfx deletion abolished the maintenance of hematopoietic stem cells (Galan-Caridad *et al*, 2007). Huang *et al* (2009) reported that *ZFX* was upregulated in side-population (SP) cells in oesophageal cancer, and that SP cells possessed CSC characteristics of oesophageal carcinoma. Therefore, we expected that the abundant expression of *ZFX* in cancer cells or in BM host cells might have crucial roles in the maintenance of human CSCs or niche cells, respectively, and their development of the metastatic phenotype.

Direct binding between *miR-144* and *ZFX* by luciferase assay and by western blotting

We investigated direct binding of *miR-144* and the *ZFX* gene by luciferase assay. In five GC cell lines (MKN45, MKN7, AZ521, MKN74, and NUGC4), we observed reduction of the luciferase activity in the pre-*miR-144*-treated lines in comparison with lines treated with the scrambled control (0.53-, 0.69-, 0.45-, 0.51-, and 0.55-fold, respectively) with statistical significance in each experiment (Figure 1A).

In addition, we performed western blotting assay for disclosing the inhibitory effect of *miR-144* on ZFX protein expression (Figure 1B). The average *ZFX* expression in parent cells, normal control cells with 10 μM of control vector, pre-*miR144* 10 μM, normal control cells with 30 μM of control vector, and pre-*miR144* 30 μM were 6.46E-01, 1.14E + 00, 7.58E-01, 1.14E + 00, and 1.37E-01, respectively. There were significant difference between pre-miR and control vector in each concentration.

Clinicopathological significance of *miR-144* and the target protein *ZFX*

In clinicopathological analysis, there was no significant association between *miR-144* expression and any clinicopathological variable. The expression ratios comparing values to the universal internal control (*miR-144* RNU6B) were 4.24 ± 4.51 for stage I and 2.33 ± 2.47 for stage IV (Figure 2A and Table 1A). These results indicated diminished *miR-144* expression during progression of disease from stage I to stage IV in GC ($P = 0.02$). On the other hand, the expression of *ZFX* gene increased with progression of the disease ($P = 0.0058$). The expression ratios for *ZFX*/*GAPDH* were as follows: 4.13 ± 5.17 (stage I), 6.64 ± 5.39 (stage II), 17.8 ± 5.22 (stage III), and 27.4 ± 5.12 (stage IV; Figure 2B and Table 1B).

Table 1A Clinicopathological significance of *miR-144* expression in BM from GC cases

	Low expression group ($n = 99$)		High expression group ($n = 99$)		
Factors	**Number**	**%**	**Number**	**%**	***P*-value**
Age (mean ± s.d.)	62.1 ± 1.22		61.6 ± 1.22		0.77
Sex					0.21
Male	65	65.7	73	73.7	
Female	34	34.3	26	26.3	
Tumour stage					0.054
m, sm	29	29.3	42	42.4	
mp, ss, se, si	70	70.7	57	57.6	
Lymph node metastasis					0.56
Absent	37	37.4	41	41.4	
Present	62	62.6	58	58.6	
Lymphatic invasion					0.78
Absent	44	44.4	46	46.5	
Present	55	55.6	53	53.5	
Venous invasion					0.32
Absent	72	72.7	78	78.8	
Present	27	27.3	21	21.2	
Peritoneal dissemination					1
Absent	95	96	95	96	
Present	4	4	4	4	
Liver metastasis					1
Absent	98	98.9	98	98.9	
Present	1	1.1	1	1.1	
Distant metastasis					0.3
Absent	98	98.9	96	97	
Present	1	1.1	3	3	
TNM stage					0.049*
I	26	26.3	39	39.4	
II, III, IV	73	73.7	60	60.6	

Abbreviations: BM = bone marrow; GC = gastric cancer; m = mucosa; mp = muscularis propria; se = serosa exposed; si = serosa infiltrating; sm = submucosa; ss = subserosa; TNM = tumour, node, metastasis. *$P<0.05$, there is a statistical significance by Student's *t*-test.

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Table 1B Clinicopathological significance of *ZFX* expression in BM from GC cases

Clinicopathological factors	Status	*n*	Expression ratio ZFX/GAPDH	*P*-value
Tumour size				0.0499*
	<5 cm	92	8.80 ± 4.00	
	⩾5 cm	113	19.4 ± 3.61	
Depth of tumour invasion				0.0107*
	m, sm	60	3.99 ± 4.92	
	mp, ss, se, si	145	19.1 ± 3.17	
Lymph node metastasis				0.0499*
	Absent	67	7.06 ± 4.70	
	Present	138	18.3 ± 3.27	
Lymphatic permeation				0.871
	Absent	80	13.4 ± 4.34	
	Present	124	15.6 ± 3.49	
Venous permeation				0.93
	Absent	138	14.6 ± 3.31	
	Present	66	15.0 ± 4.78	
Peritoneal dissemination				0.0102*
	Absent	162	11.1 ± 3.00	
	Present	43	28.1 ± 5.82	
Clinical stage				0.0101*
	I	52	4.13 ± 5.25	
	II	49	8.70 ± 5.41	
	III	51	17.8 ± 5.30	
	IV	53	27.4 ± 5.20	

Abbreviations: BM = bone marrow; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; GC = gastric cancer; m = mucosa; mp = musclaris propria; se = serosa exposed; si = serosa infiltrating; sm = submucosa; ss = subserosa. *$P < 0.05$, there is a statistical significance by Student's *t*-test.

Use of MACS fractionation to analyse BM cells with abundant *miR-144* expression in GC patients

To determine whether *miR-144* was expressed in cancer cells or host BM-derived cells, we performed cell sorting of BM by MACS (Figure 3A). Quantitative RT–PCR analysis showed the average *miR-144* expression per one cell in six GC cases of stage I and three cases of stage IV (Figure 3C). As we showed in this figure, the expression of *miR-144* per cell was significantly higher in BM3 fraction of GC cases of stage I than that of GC cases of stage IV ($P = 0.0051$). Therefore, diminished expression of *miR-144* was supposed to be originated from disseminated tumour cells.

In normal healthy sample, *ZFX* expression was 1.1 times higher in BM3 fraction than other cellular fractions, such as BM1 and BM2 (Figure 3B). However, the difference was really small among them. Therefore, the difference of *miR-144* expression might be provoked by the presence of disseminated tumour cells in BM in GC cases.

Clinicopathological significance of *miR-144* expression in primary GC

Diminished *miR-144* expression was observed in primary GC with severe tumour invasion, whereas higher *miR-144* expression was observed in GC patients with less pronounced invasion (Table 2A). There was a significant association between them (Fisher's exact test, $P = 0.037$). In fact, higher *miR-144* expression was observed in stage I and II GC patients, whereas lower *miR-144* expression was seen in stages III and IV (Fisher's exact test, $P = 0.016$).

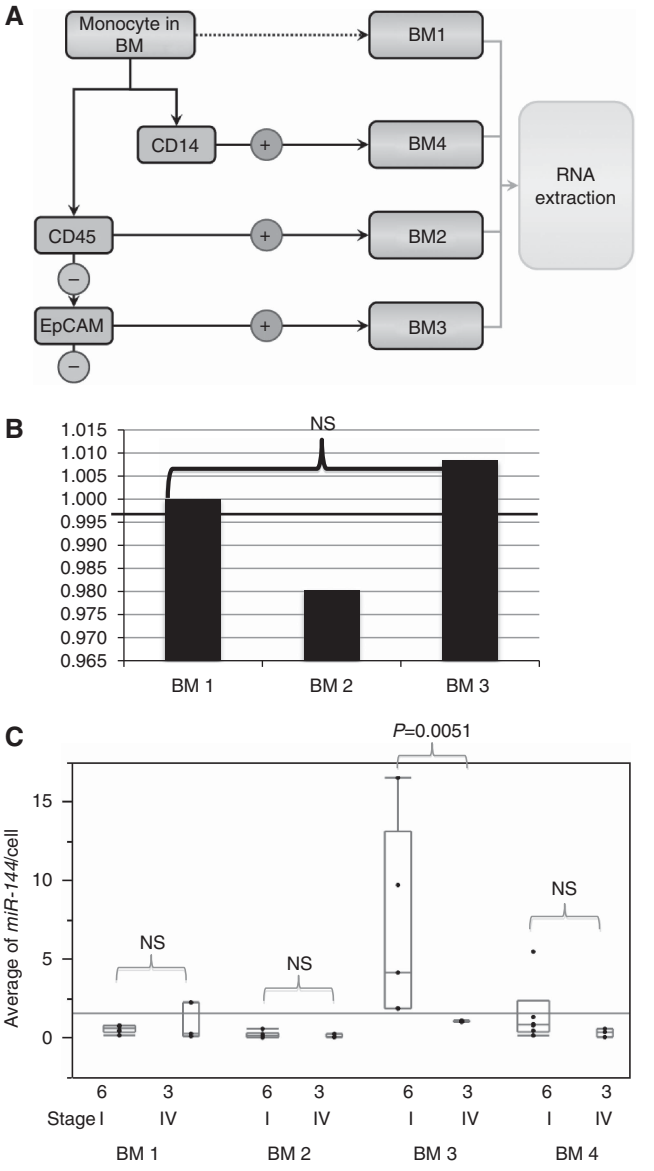

Figure 3 Determining the origin of *miR-144*-expressing cells from nine representative GC patients with distant metastasis. (**A**) We sorted BM-derived monocytes. The fraction was enriched for monocytes (6×10^7) from 15 ml of BM for BM1, BM2, and BM3 fractions. The BM2 consisted primarily of lymphocytes (2.8×10^7), and fraction BM3 was the $EpCAM^+$ and $CD45^-$ fraction, circulating epithelial cells (1.9×10^6). Another BM-derived monocytes (6×10^7) from 15 ml was stored for BM1 and sorted with antibodies $CD14^+$. The BM4 contained macrophages and monocytes (7.5×10^5). (**B**) *miR-144* expression in BM fractions in normal healthy donor sample. *miR-144* expression was 1.1 times higher in BM3 fraction than other cellular fractions, such as BM1 and BM2. However, the difference was really faint among them. (**C**) Quantitative RT–PCR analysis of the average *miR-144* expression per one cell in six GC cases of stage I and three cases of stage IV. As we showed in this figure, the expression of *miR-144* per cell was higher in BM3 fraction of GC cases of stage I than that of GC cases of stage IV significantly ($P = 0.0051$).

With regard to the prognostic significance of *miR-144* (Figure 4), 46 cases of GC with diminished *miR-144* in primary tissues had a significantly worse overall survival rate than 47 patients with higher levels of *miR-144* expression (by log-rank test, $P = 0.004$). However, the evaluation of *miR-144* was not an independent prognostic marker of GC cases with statistical significance by multivariate analysis (Table 2B).

Table 2A Clinicopathological significance of microRNA144 expression in primary gastric cancer cases

	***miR-144* primary gastric cancer**		
	High expression $n = 47$	**Low expression** $n = 46$	***P*-value**
Age	67.2 ± 10.8	62.9 ± 12.9	0.08
Gender			0.2
Male	29	34	
Female	18	12	
Tumour size			0.08
< 5 cm	24	15	
5 cm ⩽	22	29	
Depth of tumour invasion			0.037[a]
m, sm	13	5	
mp, ss, se, si	34	41	
Lymph node metastasis			0.21
Absent	18	12	
Present	29	34	
Lymphatic permeation			0.11
Absent	16	9	
Present	31	37	
Venous permeation			0.09
Absent	37	29	
Present	10	17	
Peritoneal dissemination			0.39
Absent	40	36	
Present	7	10	
Clinical stage			0.016[a]
I,II	29	17	
III,IV	18	29	

There is a statistical significance (Fisher's exact test, $P = 0.037$). Gastric cancer cases with advanced stage (III and IV) indicated much lower mi-R144 expression than stage I and II cases with statistical significance (Fisher's exact test, $P = 0.016$). [a]Gastric cancer with lower invasion showed significantly higher miR-144 expression.

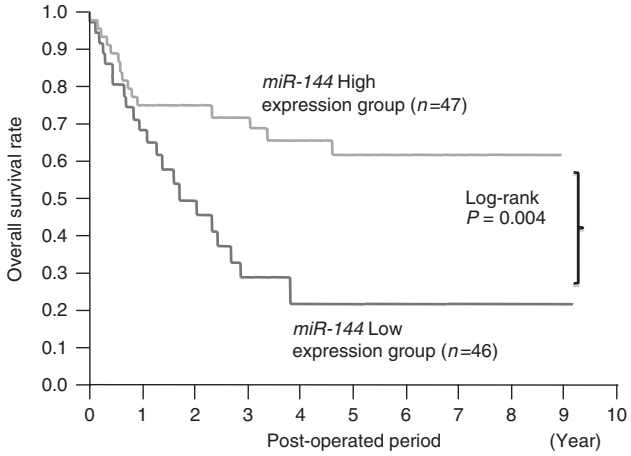

Figure 4 Comparison of the prognosis between the high and low *miR-144* expression groups by Kaplan–Meier method.

Table 2B Univariate and multivariate analysis of the clinicopathological factors and *miR-144* expression in primary gastric cancer cases for overall survival

Clinicopathological variables	**Univariate analysis**			**Multivariate analysis**		
	RR	**95% CI**	***P*-value**	**RR**	**95% CI**	***P*-value**
Gender	0.9	0.66–1.2	0.5	—	—	—
Depth (T1, T2, T3/T4)	2.59	1.65–4.71	<0.0001	1.03	0.99–1.07	0.08
Lymph node metastasis	4.05	2.45–8.21	<0.0001	2.97	1.58–7.58	0.0001
Lymphatic permeation	3.15	1.91–6.38	<0.0001	1.21	0.62–3.13	0.59
Venous permeation	1.92	1.46–2.53	<0.0001	1.05	0.69–1.6	0.79
Peritoneal dissemination	8.3	4.49–15.17	<0.0001	3.95	1.48–11.3	0.005
miR-144 (high/low)	0.71	0.5–0.99	0.0431	0.96	0.65–1.4	0.84

Abbreviations: RR = relative risk ratio; CI = confidence interval.

In vitro administration of *miR-144* inhibits *ZFX* protein and increases susceptibility to 5-FU

ZFX is believed to maintain the stemness of embryonic stem cells, whereas resistance to chemotherapy is considered to be one of the characteristics of stemness in cancer cells. In this study, we have shown that *ZFX* is directly targeted by *miR-144*. Therefore, to confirm the functional relationship between *miR-144* and *ZFX*, we investigated the effect of *miR-144* restoration on chemotherapy resistance. Anti-tumour effects of 5-FU ($0\,\mu g\,ml^{-1}$, $1.0\,\mu g\,ml^{-1}$, and $10.0\,\mu g\,ml^{-1}$) treatments were evaluated by cell proliferation (MTT) assays. Growth was reduced 0.94-, 0.85-, and 0.76-fold in the group treated with pre-*miR-144* in comparison with control cell lines, with statistical significance by ANOVA analysis (Figure 1B). This study indicated that the administration of *miR-144* in cancer cells increased responsiveness to chemotherapy.

DISCUSSION

Since Karnoub *et al* (2007) reported the role of microRNAs in breast cancer metastasis, several studies have investigated whether microRNAs are regulators of the metastatic process. To establish and enhance the reliability of our microRNA microarray data, we utilised two different platforms. We found three preferentially downregulated microRNAs in GC patients' BM in stage IV compared with stage I. Among these, *miR-144* showed the best FDR value. In addition, quantitative RT–PCR assays validated *miR-144* as a candidate for further analysis.

For analysis of the gene expression array, we applied 3D gene array (Iwano *et al*, 2010). This technological innovation enabled us to identify specific molecules even from tiny amounts of materials. Among more than 600 predicted *miR-144* target genes, the microarray validated five (*ZFX*, *FOSB*, *SUCLA2*, *LSM14A*, and *HDHD2)*. Recently, CSC is believed to have an important role in forming cancer metastasis; therefore, we have focused on genes in terms of the word listed among these five genes. In general, considering CSCs, several biological phenotypes should be proven, such as tumorigenesis, expressing specific cell surface antigens, dormant cell cycle, and resistant against treatment. Among those features, chemotherapy resistance is considered to be one of the most consecutive features, and we have focused on it in the current study. The effect of pre-*miR144* for recovering susceptibility to 5-FU was observed in MKN45 GC cell (Figure 1C); however, the alteration of susceptibility to chemotherapy rescued by *miR-144* was moderate in comparison with control cells and parent cells. The direct inhibition of *ZFX* by *miR-144* in five GC cell lines was proven distinctively by luciferase assay (Figure 1A); however, the actual protein inhibition of ZFX was moderate by *miR-144* (Figure 1B). We speculated that ZFX expression was probably rescued by other compensated systems to preserve the role of this

critical gene, which disclosed the moderate change in sensitivity to 5-FU in GC cells in the current study (Figure 1C). *ZFX* was a cancer stem-cell-related gene in terms of chemotherapy resistance; however, this gene was partially inhibited by *miR-144* effect. Therefore, we should expect the role of *miR-144* expression in BM not as a mimic microRNA to rescue the susceptibility to chemotherapy, but as an indicator of the poorer prognosis of GC cases.

In a previous study, we showed that predicting GC metastasis or recurrence could not be achieved simply by detecting circulating tumour cells. As for the *bona fide* factors contributing to metastasis, we must consider the presence of circulating (disseminated) tumour cells and BM-derived progenitor cells at premetastatic sites. As a matter of fact, Lyden *et al* (2001) highlighted the role of host BM-derived cells in the development of metastasis after curative surgery. We previously reported the importance of the simultaneous presence of disseminated tumour cells and host cells expressing *VEGFR-1*, hematopoietic progenitor cells (Mimori *et al*, 2008). Therefore, in the current study, we determined in which cells expression of *miR-144* was mainly diminished and *ZFX* upregulated in BM during progression of GC.

Here we showed that *miR-144* expression was decreased in stage IV GC, but the expression of *miR-144* was retained in stage I GC (Figure 4). Therefore, we disclosed that the loss of *miR-144* was specifically observed in epithelial cell fractions in BM. In our previous study, we clarified the existence of cancer cell clusters in BM even in stage I GC cases (Mimori *et al*, 2008); therefore, we validated that the loss of *miR-144* expression in ITC fraction in BM might have a critical role in GC cancer progression. We also found that primary GC tumours with a relatively low level of *miR-144* expression showed a poorer prognosis than GC cases with a high level of *miR-144* expression. On the other hand, one of the target molecules of *miR-144*, *ZFX*, was expressed at higher levels in BM at stage I in comparison with stage IV. Moreover, restoration of *miR-144* expression in cancer cell lines restored the cells' sensitivity to chemotherapy (Figure 1B), suggesting a key role of *miR-144* target genes in the development of GC's resistance to chemotherapy resulting from ZFX upregulation. This *in vitro* finding supported our current hypothesis that the *miR-144-ZFX* pathway has an important role primarily in disseminated cancer cells, and not in host BM cells in GC.

We speculated that cancer cells' abundant expression of *ZFX* in advanced cases means that *ZFX* might have a crucial role in the maintenance of CSCs in BM. As for the role of *ZFX* as a regulator of CSCs, recent reports are intriguing. In non-malignant embryonic stem cells, Cellot and Sauvageau (2007) described the transcriptional role of Zfx in the self-renewal/maintenance of both embryonic stem cells and hematopoietic stem cells (Galan-Caridad *et al*, 2007). Using a new software programme, Ouyang *et al* (2009) identified *ZFX* as one of 12 transcription factors upregulated in embryonic stem cells (Ouyang *et al*, 2009). On the other hand, in malignant cells, Huang *et al* (2009) reported that *ZFX*, two ATP-binding cassette (ABC) transporter genes, three Wnt, and two Notch signal pathway-related genes were upregulated in SP cells from two oesophageal cancer cells. In the current study, we demonstrated that in cells with low *miR-144* expression, *ZFX* expression was elevated and the cells were chemotherapy-resistant, presumably because those cancer cells expressed ABC transporter genes at high levels. Our study is the first to demonstrate that the loss of *miR-144* expression by disseminated cancer cells and their resultant high level of *ZFX* expression is critically important in the progression of GC in the marrow. We speculate that only *ZFX*-expressing cancer cells could survive and prepare for progression of GC.

In conclusion, we demonstrated that low *miR-144* expression in cancer cells metastasised to the marrow is associated with GC progression. Decreased expression of *miR-144* correlated with progression of the disease. We expected that reestablishing *miR-144* might be effectively used in BM to restore GC's chemosensitivity to 5-FU through its direct targeting of *ZFX* in the future. The *ZFX* gene, an X-linked zinc finger protein, has crucial roles in the maintenance of haematopoietic stem cells and the stemness of cancer cells.

ACKNOWLEDGEMENTS

We thank T Shimooka, K Ogata, M Kasagi, Y Nakagawa, and T Kawano for their technical assistance. We would really appreciate Dr H Iinuma for providing data of cell sorting in bone marrow and Professor Carlo Croce for microarray analysis in Columbus, OH, USA. This work was supported in part by the following grants and foundations: CREST, Japan Science and Technology Agency (JST); Japan Society for the Promotion of Science (JSPS) Grant-in-Aid for Scientific Research, grant numbers 20390360, 20591547, 20790960, 21591644, 21791295, 21791297, 215921014, and 21679006; and the Funding Program for Next Generation World-Leading Researchers (LS094); and NEDO (New Energy and Industrial Technology Development Organization) Technological Development for Chromosome Analysis.

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

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