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

S Akiyoshi1,2, T Fukagawa3,4, H Ueo1,2, M Ishibashi1, Y Takahashi1, M Fabbri4,6, M Sasako3, Y Maehara2, K Mimori1 and M Mori*,2,5

1Department of Surgery, Kyushu University Beppu Hospital, 4546 Tsurumihara, Beppu 874-0838, Japan; 2Department of Surgical and Science, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-Ku, Fukuoka 812-8582, Japan; 3Gastric Surgery Division, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan; 4Department of Molecular Virology, Immunology and Medical Genetics, Comprehensive Cancer Center, Ohio State University, Columbus, OH 43210, USA; 5Department of Gastroenterological Surgery, Osaka University, Graduate School of Medicine, 2-2 Yamadakou, Suita, Osaka 565-0871, Japan; 6These authors contributed equally in this study.

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 of whether 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 interaction of the microRNA expression in diverse cells (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.

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; Vison et al, 2007; Garzon et al, 2008; Liu et al, 2008a; 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...
MIR-144-ZFX in bone marrow in gastric cancer
S Akiyoshi et al

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, Target-Scan, 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 °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; Iwashashi 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 × 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 μM dithiothreitol, and 100 units of Maloney leukaemia virus RT (BRL, Tokyo, Japan). The mixture was incubated at 37 °C for 60 min, heated to 95 °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 microRNAs 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 conforming 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 × RT buffer, 0.25 nmol 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 °C, 30 min at 42 °C, 5 min at 85 °C, and then maintained at 4 °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 CTA TGG 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 MgCl₂. 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 log₂ data (BG substitution (LOG₂)). 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 electro-blotted 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.
The false discovery rate (FDR) value: (predictive pseudo positive number)/(number of genes satisfied with the threshold) = (number of genes to calculate) \times (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 Table 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 (miRanda, TargetScan, and Pictar). We also undertook quantitative comparisons of gene expression in patients with stage IV and stage I GC. Five genes (ZFX, POSB, 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...
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 30 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

| Factors                      | Low expression group (n = 99) | High expression group (n = 99) | P-value |
|------------------------------|-------------------------------|-------------------------------|---------|
| Age (mean ± s.d.)            | 62.1 ± 1.22                   | 61.6 ± 1.22                   | 0.77    |
| Sex                          |                               |                               | 0.21    |
| Male                         | 65                            | 65.7                          |         |
| Female                       | 34                            | 34.3                          |         |
| Tumour stage                 |                               |                               |         |
| m, sm                        | 29                            | 29.3                          | 0.054   |
| Tumour stage                 |                               |                               |         |
| m, sm                        | 29                            | 29.3                          | 0.054   |
| Lymph node metastasis        |                               |                               | 0.56    |
| Absent                       | 37                            | 37.4                          |         |
| Present                      | 62                            | 62.6                          |         |
| Venous invasion              |                               |                               | 0.32    |
| Absent                       | 72                            | 72.7                          |         |
| Present                      | 27                            | 27.3                          |         |
| Peritoneal dissemination      |                               |                               | 1       |
| Absent                       | 95                            | 96                            |         |
| Present                      | 4                             | 4                             |         |
| Liver metastasis             |                               |                               | 1       |
| Absent                       | 98                            | 98.9                          | 0.049*  |
| Present                      | 1                             | 1.1                           |         |
| Distant metastasis           |                               |                               | 0.3     |
| Absent                       | 98                            | 98.9                          |         |
| Present                      | 1                             | 1.1                           |         |
| TNM stage                    |                               |                               |         |
| I                            | 26                            | 26.3                          |         |
| II, III, IV                  | 73                            | 73.7                          | 0.049*  |

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.

© 2012 Cancer Research UK
British Journal of Cancer (2012) 107(8), 1345 – 1353
Molecular Diagnostics

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

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

Clinical pathological 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).

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).
and 10.0 in cancer cells increased responsiveness to chemotherapy.

the group treated with pre-

(Figure 1B). This study indicated that the administration of

microRNAs are regulators of the metastatic process. To establish

breast cancer metastasis, several studies have investigated whether

et al

Since Karnoub

DISCUSSION

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 μg ml⁻¹, 1.0 μg ml⁻¹, and 10.0 μg ml⁻¹) treatments were evaluated by cell proliferation (MTT) assays. Growth was reduced 0.94-, 0.85-, and 0.76-fold in

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

| miR-144 primary gastric cancer | High expression | Low expression | P-value |
|-------------------------------|---------------|---------------|--------|
| Age                           |               |               | 0.08   |
| Gender                        | 67.2 ± 10.8   | 62.9 ± 12.9   |        |
| Tumour size                   |               |               | 0.08   |
| <5 cm                         | 24            | 15            |        |
| ≥5 cm                         | 22            | 29            |        |
| Depth of tumour invasion      |               | 0.037a        |        |
| 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                |               | 0.016a        |        |
| II                            | 29            | 17            |        |
| III                           | 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 miR-144 expression than stage I and II cases with statistical significance (Fisher’s exact test, P = 0.016). *Gastric cancer with lower invasion showed significantly higher miR-144 expression.

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 RR 95% CI P-value | Multivariate analysis 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                | 2.97 1.58–7.58  0.0001                  |
| Lymph node metastasis         | 4.05 2.45–8.21  <0.0001                | 2.1 0.62–3.13  0.59                    |
| Venous permeation             | 1.92 1.46–2.53  <0.0000                | 1.05 0.69–1.6  0.79                    |
| Peritoneal dissemination      | 8.3 4.49–15.17  <0.0000                | 3.95 1.48–11.3  0.005                   |
| miR-144 (high/low)            | 0.71 0.5–0.99  0.0141                  | 0.96 0.65–1.4  0.84                    |

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

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

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
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 osteoskeletal 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, 21592014, 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)

REFERENCES

Alix-Panabieres C, Riethdorf S, Pantel K (2008) Circulating tumor cells and bone marrow micrometastasis. Clin Cancer Res 14: 5013–5021
Bottoni A, Zatelli MC, Ferracin M, Tagliati F, Piccin D, Vignali C, Calin GA, Negrini M, Croce DM, Degli Uberti EC (2007) Identification of differentially expressed microRNAs by microarray: a possible role for microRNA genes in pituitary adenomas. J Cell Physiol 210: 370–377
Braun S, Pantel K, Muller P, Janni W, Hepp F, Kentenich CR, Gastroth S, Wischmull A, Dimpl D, Kindermann G, Riethmuller G, Schlimok G (2000) Cytokeratin-positive cells in the bone marrow and survival of patients with stage I, II, or III breast cancer. N Engl J Med 342: 525–533
Braun S, Vogl FD, Naume B, Janni W, Osborne MP, Coombes RC, Schlimok G, Diet JF, Gerber B, Gebauer G, Pierja NT, Marth C, Orizio D, Widjwang G, Solomon P, Kudr G, Strobl B, Fehm T, Wong GY, Bliss J, Vincent-Salomon A, Pantel K (2005) A pooled analysis of bone marrow micrometastasis in breast cancer. N Engl J Med 353: 793–802
Calin GA, Croce CM (2007) Investigation of microRNA alterations in leukemias and lymphomas. Methods Enzymol 427: 193–213
Calin GA, Liu CG, Sevignani C, Ferracin M, Felli N, Dumitru CD, Shimizu M, Cimmino A, Zupo S, Dono M, Dell’Aquila ML, Alder H, Rassenti L, Kipps TJ, Bullrich F, Negrini M, Croce CM (2004) MicroRNA profiling reveals distinct signatures in B cell chronic lymphocytic leukemias. Proc Natl Acad Sci USA 101: 11755–11760
Cellot S, Sauvageau G (2007) Zfx: at the crossroads of survival and self-renewal. Cell 129: 239–241
Choesmel V, Arract P, Koifod H, Thiery JP, Blin N (2004) A relevant immunomagnetic assay to detect and characterize epithelial cell adhesion molecule-positive cells in bone marrow from patients with breast carcinoma: immunomagnetic purification of micrometastases. Cancer 101: 693–703
Dirks P (2010) Cancer stem cells: invitation to a second round. Nature 466: 41
Engel H, Kleesies C, Friedrich J, Breidenbach M, Kallborn A, Schondorf T, Kolhagen H, Mallmann P (1999) Detection of circulating tumour cells in patients with breast or ovarian cancer by molecular cytogentic. Br J Cancer 81: 1165–1173
MIR-144-ZFX in bone marrow in gastric cancer
S Akiyoshi et al

Galan-Caridad JM, Harel S, Arenzana TL, Hou ZE, Doetsch FK, Minny LA, Reizis B (2007) Zfx controls the self-renewal of embryonic and hematopoietic stem cells. *Cell* **129**: 345–357

Garzon R, Volinia S, Liu CG, Fernandez-Cymering C, Palumbo T, Pichiiorri F, Fabbri M, Coomes B, Alder H, Nakamura T, Flomenberg N, Marcucci G, Calif GA, Kornblau SM, Kantarjian H, Bloomfield CD, Andreff M, Croce CM (2008) MicroRNA signatures associated with cytogenetics and prognosis in acute myeloid leukemia. *Blood* **111**: 3183–3189

Gottardo F, Liu CG, Ferracin M, Calif GA, Fassan M, Bassi P, Sevignani C, Byrne D, Negrini M, Pagano F, Gomella LG, Croce CM, Baffa R (2007) MicroRNA profiling in kidney and bladder cancers. *Ourl Oncol* **26**: 323–330

Huang D, Gao Q, Guo L, Zhang C, Jiang W, Li H, Wang J, Shi Y, Lu SH (2009) Isolation and identification of cancer stem-like cells in esophageal carcinoma cell lines. *Stem Cells Dev* **18**: 465–473

Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, Sabbioni S, Magri E, Pedriali M, Fabbri M, Campiglio M, Menard S, Palazzo JP, Rosenberg A, Musiani P, Volinia S, Nenci I, Calif GA, Querzoli P, Negrini M, Croce CM (2005) MicroRNA gene expression deregulation in human breast cancer. *Cancer Res* **65**: 7065–7070

Ito T, Tanaka E, Kadowaki T, Kan T, Higashiyama M, Shiojima S, Tomoda S, Myoumoto A, Akiyama H, Nobumasa H, Matsumoto S, Miyamoto S, Mitsumori M, Sato F, Watanabe G, Itami A, Meltzer SJ, Tsujiomi G, Shimada Y (2007) An ultrasensitive new DNA microarray chip provides gene expression profiles for preoperative esophageal cancer biopsies without RNA amplification. *Oncology* **73**: 366–375

Iwahashi H, Kitagawa E, Suzuki Y, Ueda Y, Ishizawa YH, Nobumasa H, Kuboki Y, Hosoda H, Iwashishi Y (2007) Evaluation of toxicity of the mycoctoxin citrinin using yeast ORF DNA microarray and Oligo DNA microarray. *BMC Genomics* **8**: 95

Iwano S, Ichikawa M, Takizawa S, Hashimoto H, Miyamoto Y (2010) Identification of AhR-regulated genes involved in PAH-induced immunotoxicity using a highly-sensitive DNA chip, 3D-Gene Human Immunity and Metabolic Syndrome 9k.

Kaplan RN, Riba RD, Zacharoulis S, Bramley AH, Vincent L, Kottke ML, Thiele J, Fischer R (1994) Magnetic activated cell sorting (MACS) – a new immunomagnetic method for megakaryocytic cell isolation: Clinical quantitation of diagnostic and predictive gene expression levels in follicular and diffuse large B-cell lymphoma by RT-PCR gene expression profiling. *Blood* **109**: 3922–3928

Schetter AJ, Reizis B (2007) Zfx controls the self-renewal of embryonic and hematopoietic stem cells. *Nature* **449**: 425–436

Sakhinia E, Glennie C, Hoyer JA, Menasse LP, Brady G, Miller C, Radford JA, Byers RJ (2007) Clinical quantitation of diagnostic and predictive gene expression levels in follicular and diffuse large B-cell lymphoma by RT-PCR gene expression profiling. *Proc Natl Acad Sci USA* **104**: 22–30

Visone R, Pallante P, Vecchione A, Giombella R, Ferracin M, Ferraro A, Volinia S, Coluzzi S, Leone V, Borbone E, Liu CG, Petrocca F, Troncone G, Calif GA, Scarpa A, Colato C, Tallini G, Santoro M, Croce CM, Fusco A (2007) Specific microRNAs are downregulated in human thyroid anaplastic carcinomas. *Oncogene* **26**: 7590–7595

Yanaihara N, Caplen N, Bowman E, Seike M, Kamamoto K, Yi M, Stephens RM, Okamoto A, Yokota J, Tanaka T, Calif GA, Liu CG, Croce CM, Harris CC (2006) MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. *Jama* **299**: 267–275

This work is published under the standard license to publish agreement. After 12 months the work will become freely available and the license terms will switch to a Creative Commons Attribution-NonCommercial-Share Alike 3.0 Unported License.