Biomarkers, 2012; 17(5): 463–470

ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.3109/1354750X.2012.686061

informa
healthcare

RESEARCH ARTICLE

miRNA expression profiling for identification of potential breast cancer biomarkers

Kjersti Tjensvoll[1], Kine N. Svendsen[1], James M. Reuben[2], Satu Oltedal[1], Bjørnar Gilje[1], Rune Smaaland[1], and Oddmund Nordgård[1]

[1]*Department of Haematology and Oncology, Stavanger University Hospital, Stavanger, Norway and* [2]*Department of Hematopathology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA*

Abstract

To identify micro RNA (miRNA) biomarker candidates for early detection of breast cancer and detection of minimal residual breast cancer, we performed miRNA expression profiling in pooled RNA samples from breast tumors, and from bone marrow mononuclear cells, peripheral blood mononuclear cells and plasma from healthy controls. We found substantially higher levels of five miRNAs in the breast tumors compared to the normal samples. However, validation of these miRNA levels, and seven other candidates selected from the literature, in individual samples from healthy controls and patients with non-metastatic breast cancer did not suggest further examination of their biomarker potential.

Keywords: Breast cancer, minimal residual disease, blood, plasma, CTC, DTC, miRNA

Introduction

Hematogenous spread of cancer cells into distant organs and their subsequent growth into overt metastases is the main cause of death among breast cancer patients (Eccles & Welch 2007). This has led to a significant interest in identifying markers for early detection of disseminated and circulating tumor cells. Although our understanding is still limited with regard to the molecular and cellular mechanisms driving the metastatic process, the discoveries of small regulatory RNA molecules named micro RNAs (miRNAs) opened a new era for research on cancer metastasis.

Micro RNAs are small non-coding RNA molecules, typically 18–25 nucleotides long, known to control development, differentiation, cellular proliferation, programmed cell death, carcinogenesis and metastasis (Shi et al. 2010). They regulate gene expression at the post-transcriptional level by two strategies: (i) suppression of protein translation or (ii) cleavage of target messenger RNAs (mRNAs) to induce their degradation (Jackson & Standart 2007). The miRNA expression is tissue-specific, and altered miRNA expression patterns have been shown to correlate with molecular subtypes of breast cancer (Blenkiron et al. 2007). Furthermore, several miRNAs are shown to be differentially expressed between primary tumors and related metastatic lymph nodes (Baffa et al. 2009). Altered expression of several circulating miRNAs has also been identified in breast cancer patients as compared to healthy individuals (Heneghan et al. 2010; Roth et al. 2010; Zhao et al. 2010). Heneghan et al. (2010) showed that the level of both miR-195 and let-7a were significantly higher in whole blood from breast cancer patients, and that the miRNA blood levels decreased in patients postoperatively (Heneghan et al. 2010). Roth et al. (2010) demonstrated that miR-155 in serum significantly discriminated non-metastatic (M0) breast cancer patients from healthy individuals and that this miRNA, among others, also correlated with the presence of overt metastasis (Roth et al. 2010). All these results suggest that miRNAs could be useful as potential

Address for Correspondence: Kjersti Tjensvoll, Laboratory for Molecular Biology, Department of Haematology and Oncology, Stavanger University Hospital, N-4068 Stavanger, Norway. Tel: +47 47809206. E-mail: ktje@sus.no; tjensvollk@gmail.com

(Received 14 March 2012; revised 13 April 2012; accepted 14 April 2012)

surrogate markers of both early disease and prognosis in breast cancer.

Hence, the aim of the present study was to perform miRNA expression profiling to identify miRNAs that are highly expressed in primary tumors and to contrast them with miRNAs that are commonly not expressed or expressed at a low level in bone marrow mononuclear cells (BM-MCs), peripheral blood mononuclear cells (PBMCs) and plasma of healthy individuals. Differentially expressed miRNAs, and several miRNAs suggested in previous studies, were further explored as surrogate markers of early disease and prognosis in hematopoietic tissue samples from breast cancer patients with a median follow-up time of 98 months (>8 years).

Patients and methods

Patients

The patients included in this study were consecutively recruited during the years 1998–2000 as previously described (Tjensvoll et al. 2009). The patients were admitted to our hospital with primary operable, localized, invasive breast cancer (M0). Written informed consent was obtained from all participants for this project that was approved by the Regional Committees for Medical and Health Research Ethics.

BM, venous PB and plasma samples from 14 M0 breast cancer patients were analysed; randomly selecting (by computer) seven patients among those with known disease recurrence and seven among those patients without disease recurrence after 98 months (>8 years) of follow-up, from our larger patient cohort described previously (Tjensvoll et al. 2009). The seven patients with known disease recurrence corresponded to 23% of the patients with systemic relapse in the total cohort, n = 191. The patient characteristics are summarized in Table 1. BM aspirates and venous PB samples obtained from 10 healthy women, without any known cancer, constituted the control group.

Table 1. Comparison of the clinicopathological parameters according to patients with disease recurrence (Rec+), and patients without disease recurrence (Rec–).

Variable	No. of patients (n = 14)	Patients Rec+ (n= 7)	Patients Rec– (n= 7)	p value
Age				0.59
≤55	8	5	3	
>55	6	2	4	
Lymph node status				0.19
pN0	11	4	7	
pN1-2	3	3	0	
Tumor size				1.00
pT1	10	5	5	
pT2-4	4	2	2	
Tumor grade				0.42
1	5	1	4	
2	4	3	1	
3	5	3	2	
ER-status				1.00
Positive	11	5	6	
Negative	3	2	1	
PgR-status				1.00
Positive	5	3	2	
Negative	9	4	5	
Histological type				1.00
Ductal	9	5	4	
Lobular	1	0	1	
Mixedductal/lobular	3	2	1	
Others	1	0	1	

None of the breast cancer patients had evidence of distant metastases (stage M_0).
ER, estrogen receptor; PgR, progesterone receptor.

Samples

In the present study, we have analysed (i) BM samples (20 mL collected in heparin anticoagulant) obtained prior to surgery by unilateral aspiration from the posterior iliac crest under local anesthesia, (ii) venous PB (20 mL collected in heparin) samples and (iii) plasma samples. BM-MCs and PBMCs were isolated, as described previously (Shammas et al. 1999). The plasma samples were obtained by centrifugation of the PB samples at 15000 rpm for 10 min.

The miRNA expression profiling was performed on a pooled RNA sample consisting of RNA from 10 breast tumors, a pooled RNA sample consisting of RNA from 10 BM-MC samples from healthy individuals, a pooled RNA sample consisting of RNA from 10 PBMC samples from healthy individuals and a pooled RNA sample consisting of RNA from 10 plasma samples from healthy individuals. Following the miRNA profiling the expression levels of 12 potential miRNA candidates were examined in (i) five breast cancer cell lines, MCF-7, MDA-MB-361, MDA-MB-453, ZR-75-1 and T47D; (ii) 10 individual tumor samples (the same that were pooled) from M0 breast cancer patients; (iii) BM-MC, PBMC and plasma samples obtained from 10 healthy women; and (iv) BM-MC, PBMC and plasma samples from 14 randomly selected M0 breast cancer patients. An overview of the samples included in this study, and the experimental procedure is shown in Figure 1. The samples were analysed by two individuals (K.T. and K.N.S.); both being blinded to the clinical outcome of the patients.

RNA isolation

Total RNA, including miRNA, was isolated from cell line lysates and clinical samples using Qiagens "miRNeasy Mini Kit". RNA concentrations were determined by measurements on the NanoDrop 2000c instrument (Thermo Fisher Scientific Inc., Wilmington, MA, USA). The RNA quality was assessed on Bioanalyzer 2100 (Agilent Technologies Inc., Wilmington, DE, USA) using the "RNA 6000 Nano kit" and the "Small RNA kit" (Agilent Technologies).

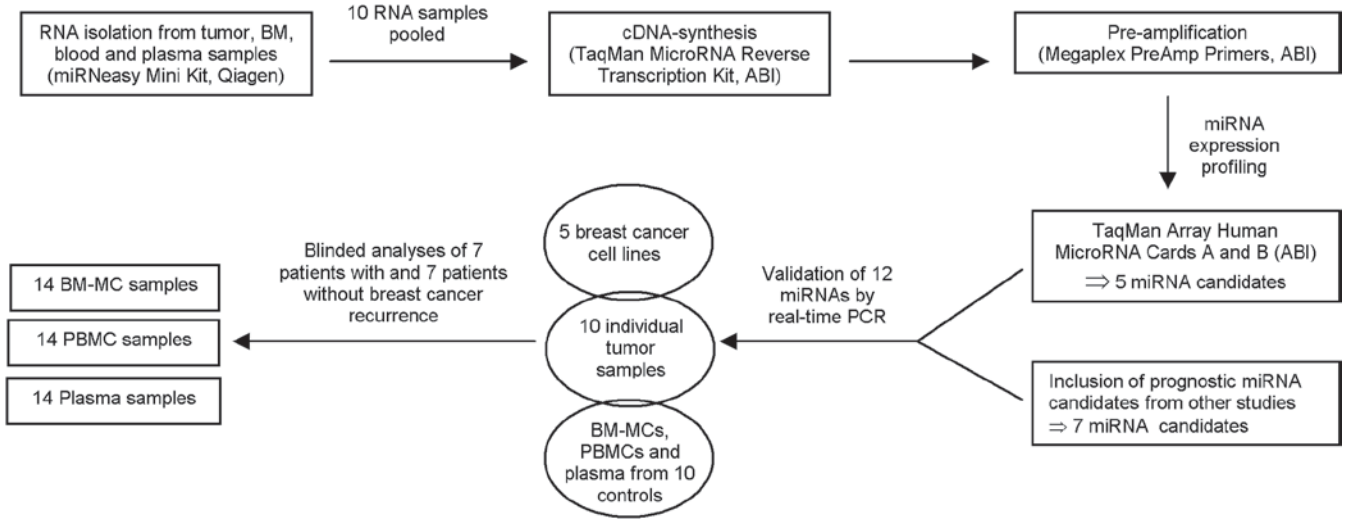

Figure 1. An overview of the samples analysed, and the experimental procedures included in the present study. BM-MC denotes bone marrow mononuclear cells while PBMC denotes peripheral blood mononuclear cells.

miRNA expression profiling

Each pool of RNA (from tumor, BM-MCs, PBMCs and plasma) in the miRNA profiling experiment contained 50 ng total RNA, which was reverse transcribed using the "TaqMan® MicroRNA Reverse Transcription Kit" with the "Megaplex RT Primers, Human Pool Set v3.0" as described by the manufacturer (Applied BioSystems, Foster City, CA, USA). To mitigate the inhibition of the PCR by heparin, the pooled plasma RNA sample was treated with Heparinase I (Sigma-Aldrich, St. Louis, MO, USA) during the reverse transcription (RT) as described by Johnson and colleagues (Johnson et al. 2003). In brief, 50 ng (3 μL) total RNA were added 0.10 μL RNAse inhibitor (20 U/μL), 0.40 μL 10× RT buffer, 0.45 μL 25 mM $MgCl_2$, 0.20 μL RNAse-free water and 0.3 μL Heparinase (1 U/μL) before the tube was incubated at room temperature for 1 h. Following the heparinase treatment, 0.20 μL 100 mM dNTPs, 0.40 μL 10× RT buffer, 0.45 μL 25 mM $MgCl_2$ and 0.80 μL 10× Megaplex RT primers were added, and the tube was incubated on ice for 5 min before the reverse transcription was performed, as recommended by the manufacturer (Applied BioSystems). After the cDNA synthesis the pooled tumor, the pooled BM-MC, the pooled PBMC and the pooled plasma samples were pre-amplified using the "Megaplex PreAmp Primers, Human Pool Set v3.0" from Applied Biosystems. After pre-amplification, the PCR products were diluted in 75 μL of 0.1× TE pH = 8, and stored at –20°C.

The miRNA expression profiling was performed by mixing 450 μl 2× TaqMan Universal PCR Master Mix (No AmpErase® UNG), 9 μL diluted PreAmp product and 441 μL dH_2O. Of this PCR mixture, 100 μL was loaded into each port on the TaqMan Array Human MicroRNA Cards A and B (Applied BioSystems). Card A consisted of unique real-time PCR assays for the more highly characterized miRNAs, while Card B contained assays for several novel miRNAs. The cards were run in a 7900HT Real-Time PCR instrument using the 384-well TaqMan Low Density Array default thermal-cycling conditions (Applied BioSystems). The RT-PCR data were analysed in the RQ Manager 1.2 program using the pooled tumor sample as a calibrator. The relative expression level of individual miRNA was represented by $\Delta C_q = C_{qmiRNA} - C_{qU6}$, as U6 RNA was included as a reference gene on the TaqMan Array Human MicroRNA Cards.

The TaqMan Array Human MicroRNA Cards A and B were searched for candidate miRNA biomarkers using two criteria: (i) the miRNAs should be highly expressed in the tumor pool, and not expressed in the BM-MC pool, the PBMC pool or the plasma pool consisting of RNA from 10 healthy individuals or (ii) the miRNAs should be highly expressed in the tumor pool, and have low expression in the BM-MC, PBMC or plasma pool consisting of RNA from 10 healthy individuals.

Reference transcript for relative quantification

Four transcripts (miR-92, miR-26b, RNU24 and RNU6) were evaluated for use as endogenous controls in this study (Wong et al. 2007), and their expression levels were determined in 15 healthy control BM-MC samples and 10 tumor samples. RNU24 and miR-92 were the most constantly expressed transcripts across the samples analysed. Due to miR-92 being a miRNA, and thus is structurally similar to the transcripts under investigation, this transcript was chosen as a reference for the further analyses.

Validation of the selected miRNA biomarker candidates by RT-qPCR in individual samples

RNA (10 ng) isolated from individual tumor samples, cell lines, BM-MCs, PBMCs and plasma was reverse transcribed to cDNA using the "TaqMan® MicroRNA Reverse Transcription Kit" with miRNA specific primers as described by the manufacturer (Applied BioSystems).

To each of the cDNA samples, the following RT-qPCR reagents (20 μL) were added: 1.0 μL 20× TaqMan

MicroRNA Assay (Table 2), 1.33 μL cDNA, 10.0 μL TaqMan 2× Universal PCR Master Mix no AmpErase UNG and 7.67 μL dH_2O. Amplification of this mixture was performed in duplicate in a LightCycler 480 (Roche Applied Science, Indianapolis, IN, USA) instrument using the following cycling conditions 95°C for 10 min; 40 cycles of 95°C for 15 s and 60°C for 60 s.

Mean C_q-values (denotes the number of cycles to reach the threshold value, previously denoted C_t and C_P (Bustin et al. 2009)) based on the two replicates analysed, were used in the calculations. The miRNA levels were normalized against miR-92 levels. Due to absent expression of some miRNAs in the cell lines and tumor samples, no calibrator was used in this experiment. Relative gene expression was calculated for each miRNA using $R = 2^{\Delta c_q}$, where $DC_q = C_q(\text{miR-92}) - C_q(\text{miR-X})$. Relative miRNA concentrations were also calculated in those cases where the miRNA was only amplified in one of the two replicates analysed. Moreover, in cases where the miRNA was expressed in healthy individuals, a threshold value was established representing the highest relative gene expression level in the control group.

Cell culture

The miRNA expression levels were evaluated in the cell lines MCF-7, MDA-MB-361, MDA-MB-453, ZR-75-1 and T47D. The MCF-7 cells were cultured in Eagle's Minimum Essential Medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich) and 1% non-essential amino acids (Sigma-Aldrich). Both MDA-MB-361 and MDA-MB-453 cells were cultured in L15 medium (Sigma-Aldrich) supplemented with 15% (MDA-MB-261) and 10% (MDA-MB-453) FBS, respectively. The ZR-75-1 cells were cultured in RPMI-1640 (Sigma-Aldrich) supplemented with 10% FBS. T47D cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich), also added 10% FBS. All the culture media were supplemented with 2 mM glutamine (Sigma-Aldrich) and 5 mL penicillin-streptomycin (Sigma-Aldrich).

Table 2. Specification for the TaqMan MicroRNA Assays (ABI) used in this study.

miRNAs	Assay ID	miRBase no.
miR-517c	001153	MI0003174
miR-512-3p	001823	MIMAT0002823
miR-214*	002293	MI0000290
miR-887	002374	MI0005562
miR-522	002413	MI0003177
miR-141	000463	MI0000457
miR-200c	002300	MI0000650
miR-205	000509	MI0000285
miR-214	002306	MI0000290
miR-10b	002218	MI0000267
miR-189	000488	MI0000485
miR-195	000494	MI0000489
miR-92	000430	MI0000093/94

Statistical analyses

The statistical analyses were performed using SPSS version 18.0 (www.spss.com) with a two-sided p value ≤0.05 considered as statistically significant. To assess whether patients with miRNA expression in PBMCs also had presence of circulating miRNAs in their plasma, a χ^2 test was performed. We also performed the Mann–Whitney U-test to compare the miRNA levels (when detectable) in (i) Tumor versus healthy controls (ii) Healthy controls versus patients and (iii) Patients with disease recurrence versus patients without recurrence.

The plots were made using the R statistical software package version 2.9.2 (www.r-project.org). Samples without miRNA expression were plotted below the limit of detection (LOD) line.

Results

Biomarker screening by miRNA expression profiling

In search for potential markers of early and minimal residual breast cancer, we performed expression profiling of 754 miRNAs in a pooled breast tumor sample, a pooled normal BM-MC sample, a pooled normal PBMC sample and a pooled normal plasma sample by TaqMan Array Human miRNA Cards. The selection of potential miRNA biomarkers was based on high expression in the pooled tumor sample, and undetectable or low expression levels in the pooled normal BM-MC, PBMC and plasma control samples. Based on these criteria, the five miRNAs miR-517c, miR-512, miR-214*, miR-887 and miR-522 were selected for further validation. These miRNAs showed high expression in the pooled tumor sample (C_q-values ranging from 21.45–23.69), and had undetectable expression in the pooled BM-MC, PBMC and plasma control samples on the TaqMan Array Human miRNA cards.

Selection of miRNA biomarker candidates from other studies

We also selected several miRNAs for validation, based on their reported relevance to breast cancer. Among these miRNAs were miR-10b, miR-195 and miR-214 (Blenkiron et al. 2007; Ma et al., 2007; Heneghan et al. 2010), and three miRNAs miR-141, miR-200c and miR-205 included in a metastatic cancer miRNA signature (Baffa et al. 2009). Furthermore, analyses of previously published micro-array data sets (Lowery et al. 2009; Velu et al. 2009) revealed miR-189 as a candidate marker. This was due to high expression in primary breast tumors (GEO:GSE15885) and low background expression in normal BM (GEO:GSE15077). When we compared the expression data from the TaqMan Array Human miRNA Cards, all the selected miRNAs, except for miR-189 (not included) and miR-195, were highly expressed in the pooled tumor sample and had low background expression in all the pooled normal control samples analysed.

Validation of the miRNA biomarker candidates in individual breast tumors and individual normal BM, PB and plasma samples

For validation of the 12 selected miRNA biomarker candidates (miR-517c, miR-512, miR-214*, miR-887, miR-522, miR-10b, miR-195, miR-189, miR-214, miR-141, mir-200c and miR-205), their expression levels were determined in individual breast tumors, cell lines and individual normal BM-MC, normal PBMC and normal plasma samples by singleplex RT-qPCR (Figures 2, 3, and S1). Six of the 12 miRNAs were expressed at relatively high levels (average C_q-values from 21.7 to 25.4) in the tumor samples, while the other candidates expressed moderate levels. In the breast cancer cell lines MCF-7, MDA-MB-361, MDA-MB-453, T47D and ZR-75-1, the levels varied from tumor-like to undetectable. The marker levels in the normal control samples were low, but not sufficiently low to suggest a biomarker potential. The highest difference in expression level was seen with miR-141, which had 2900-fold higher median tumor level compared to the highest normal plasma level. However, some miRNA candidates had undetectable levels in all or some of the hematogenous sample types, which suggested a potential role as biomarkers. MiR-887 was undetectable in both healthy BM-MC, PBMC and plasma control samples while miR-205 was undetectable in normal BM-MCs and PBMCs, miR-512 in normal BM-MCs and plasma, miR-214* in normal PBMCs, miR-214 in normal BM-MCs and miR-522 in normal plasma samples (Figures 2, 3, and S1).

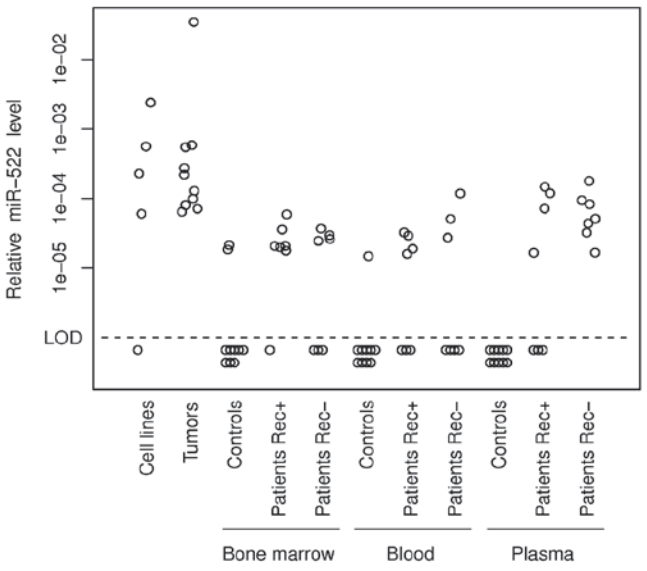

Figure 2. Relative levels of miR-522 in cell lines and primary tumors in addition to bone marrow mononuclear cells, peripheral blood mononuclear cells and plasma obtained from healthy control individuals and non-metastatic breast cancer patients. Seven of the patients analysed had experienced recurrence (Patients Rec+), while seven patients were without recurrence (Patients Rec–) after a median 98-month follow-up. Samples with undetectable levels are shown below the dashed line (LOD = limit of the detection).

Validation of the selected miRNA biomarker candidates in BM, PB and plasma samples from non-metastatic breast cancer patients

The 12 selected miRNA candidates were also validated in BM-MCs, PBMCs and plasma obtained from 14 M0 breast cancer patients (Table 1) randomly selected from a larger cohort of non-metastatic breast cancer patients (Tjensvoll et al. 2009). Seven of the M0 patients had subsequent disease recurrence, while seven had not. Potential miRNA biomarker candidates for early disease detection were expected to have higher levels in patient samples than in healthy control samples, while higher miRNA levels in the patients with recurrence would indicate possibilities as prognostic markers. Therefore, we first compared the specific miRNA levels in the patient samples with the corresponding samples from healthy controls, and found that miR-522 most frequently had elevated levels in the patient samples. Compared to the highest normal levels, 10 BM-MC samples, 7 PBMC samples and 11 plasma samples from the 14 examined breast cancer patients had elevated miR-522 levels. This corresponded to a maximum sensitivity of 79% (11/14) in plasma, which suggested plasma miR-522 as a candidate biomarker for early detection of breast cancer (Figure 2). However, the patient plasma level of miR-522 was barely detectable (C_q-values between 37 and 38), suggesting low reproducibility.

With regard to the miRNAs potential as prognostic markers, none of the miRNA biomarkers could differentiate between patients experiencing recurrence and patients without recurrence.

Discussion

We hypothesized that miRNAs could be promising new biomarkers for detection of early and minimal residual breast cancer based on the following premises: (i) miRNA expression is frequently dysregulated in cancer (Lu et al. 2005; Calin & Croce 2006; Esquela-Kerscher & Slack 2006; Blenkiron et al. 2007; Janssen et al. 2010; Andorfer et al. 2011);(ii) miRNAs appear to be tissue-specific (Lu et al. 2005); and (iii) miRNAs are exceptionally stable in plasma and serum (Mitchell et al. 2008). Our strategy was to perform miRNA expression profiling of pooled RNA samples to reveal miRNAs that were differentially expressed between breast tumors in comparison to BM-MCs, PBMCs and plasma obtained from healthy individuals. By running the TaqMan Array Human miRNA Cards, five candidate miRNA biomarkers were identified. In addition, we selected an additional seven candidate miRNA biomarkers from other studies, based on their expression in metastatic breast cancer cells or for their prognostic value in primary tumors (Blenkiron et al. 2007; Ma et al. 2007; Baffa et al. 2009; Heneghan et al. 2010). None of the 12 miRNA candidates analysed did, however, exhibit any particularly promising potential as a biomarker for early breast cancer detection or prognostic stratification after validation in a small patient cohort.

The undetectable miRNA expression level seen in some healthy control samples on the TaqMan arrays were not totally confirmed in the validation experiment of single samples. This was also the case for the high tumor levels seen for some miRNAs on the TaqMan arrays. The pre-amplification step, performed to enhance the miRNA detection on the TaqMan arrays, may explain the difference in tumor levels between the two experiments, but not the absent background expression. The pre-amplification will reduce the C_q-values of the samples by adding additional thermal cycles before the real-time PCR analysis. Although undetectable on the TaqMan arrays, some miRNAs were detected at moderate levels in the normal control samples by singleplex TaqMan RT-PCR. This might be due to a lower cDNA concentration of each sample in the profiling experiment, which reduces the chances for amplification of the less concentrated miRNAs. However, a more likely explanation is that the cDNA synthesis is more effective when only one miRNA target is being transcribed by gene-specific primers, compared to the multiplex RT reaction that was performed in the miRNA profiling experiment. In the multiplex RT reaction, there may be a competition between the transcripts, resulting in a limitation of reagents for transcription of the rarer miRNAs.

For evaluation of the miRNA candidates as markers for prognostic stratification and detection of early disease, we quantitated the miRNA levels in BM-MCs, PBMCs and plasma from 14 randomly selected M0 breast cancer patients. In these analyses, we found that none of the 12 miRNAs could distinguish patients with disease recurrence from those without. However, the low number of patients investigated suggests careful interpretation. Interestingly, Gregory and colleagues showed that miR-200c, miR-141 and miR-205, which were also evaluated in our study, were markedly down-regulated in cells that had undergone epithelial-to-mesenchymal transition (EMT) (Gregory et al. 2008). Recent evidence suggests that EMT processes are active in the CTCs (Bonnomet et al. 2011). Accordingly, miRNAs that are down-regulated during the EMT process may be poor markers for detection of CTCs with a metastatic potential.

MiR-522 was detected exclusively in patient plasma samples, and not in any of the normal samples. However, the patient plasma levels were barely detectable, indicating low reproducibility. Further validation of miR-522 in plasma samples from additional healthy control samples and breast cancer patients confirmed our concern regarding reproducibility (results not shown).

MiR-10b is strongly expressed in metastatic breast cancer cells undergoing EMT and to positively regulate cell migration, and invasion (Ma et al. 2007). Ma and colleagues also showed that 50% of metastatic (M1) breast cancer patients had elevated levels of miR-10b in their primary tumors, compared to normal breast tissue. M0 patients, on the other hand, were shown to contain decreased miR-10b tumor levels (Ma et al. 2007). MiR-10b has also been demonstrated to be differentially expressed between normal breast tissues and breast tumors, in other studies (Iorio et al. 2005). In our study of M0 breast cancer patients, miR-10b could not differentiate between patients with and without disease recurrence (Supplemental Figure S1). Our results in PBMCs are in concordance with those of Heneghan and colleagues (Heneghan et al. 2010) who showed that miR-10b levels in whole blood were within the normal range, and 71% of their patient cohort were with early-stage disease (Heneghan et al. 2010).

In the same study, Heneghan and colleagues also reported that the miR-195 level in whole blood from patients with non-metastatic breast cancer was significantly higher than in whole blood from healthy controls (Heneghan et al. 2010). These results are however – in contrast to our data – show no significant difference in the PBMC miR-195 levels between M0 breast cancer patients and healthy controls (Figure 3). On the other hand, there seems to be an inconsistency in the number of normal control samples and their miR-195 level in the figures of Heneghans report (Heneghan et al. 2010), creating uncertainty regarding the magnitude of the difference in blood miR-195 levels between patients and healthy controls. Moreover, in contrast to Heneghan et al. (2010), we present survival data with a median follow-up time of 98 months (longer than 8 years), suggesting that the miR-195 blood levels could not discriminate between breast cancer patients with and without disease recurrence.

There have been concerns that cell-free miRNAs present in the circulation might not represent metastatic

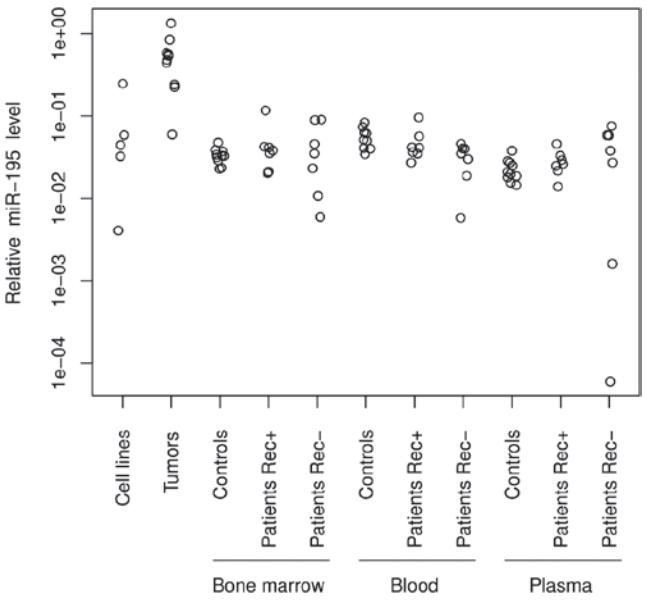

Figure 3. Relative levels of miR-195 in cell lines and primary tumors in addition to bone marrow mononuclear cells, peripheral blood mononuclear cells and plasma obtained from healthy control individuals and non-metastatic breast cancer patients. Seven of the patients analysed had experienced disease recurrence (Patients Rec+), while seven patients were without recurrence (Patients Rec–) after a median 98-month follow-up. Samples with undetectable levels are shown below the dashed line (LOD = limit of the detection).

or primary tumor tissue, and that measurements of circulating tumor cell (CTC)-associated miRNA would be preferable (reviewed in Mostert et al. 2011). On the other hand, evidence that the large majority of miRNAs in blood are present in a cell-free form has been reported, but tumor cells have also been shown to release miRNAs into the circulation (Mitchell et al. 2008). CTC-associated miRNA expression patterns can therefore differ from circulating free miRNA patterns seen in plasma. Thus, we wanted to investigate whether non-metastatic breast cancer patients with elevated levels of specific miRNAs in PBMCs also had elevated levels of the same miRNAs in their plasma samples. Assessment of the χ^2 test demonstrated that there was not a statistically significant association between PBMC miRNA status and the plasma miRNA status in the 14 patient samples analysed in our study.

Post-operative detection of disseminated and circulating tumor cells is expected to be more informative in regard to prognosis than pre-operative detection, since the passive shedding of tumor cells observed from primary tumors should be expected to end after removal of the tumor by surgery. However, we have demonstrated by specific mRNA measurements in BM-MCs, that pre- and post-operative BM sampling (3 weeks and/or 6 months after surgery) have similar prognostic value (Tjensvoll, in press). In contrast, Heneghan and colleagues have shown that the miRNA levels in blood decrease to levels comparable with healthy control subjects two weeks after resection. With regard to this, they suggest that the half-life of tumor-associated miRNAs in blood is less than 14 days, and that systemic miRNA profiling could only be utilized as non-invasive biomarkers for breast cancer (Heneghan et al. 2010; Heneghan et al. 2011). However, although the level is low, the tumor-derived miRNAs present in blood after surgery may originate from micrometastases and thus have prognostic value.

Conclusion

In the present study, we searched for potential miRNA biomarkers for early disease detection and prognostic stratification in BM-MCs, PBMCs and plasma from patients with non-metastatic breast cancer. Although the low number of patients investigated suggests careful interpretation of the results, none of the 12 miRNA candidates analysed seemed to have any potential as breast cancer biomarkers in our study. The lack of any promising trends in this first validation did not encourage us to validate these miRNAs in a larger cohort.

Declaration of interest

This study was partly funded by the Norwegian Cancer Society.

References

Andorfer CA, Necela BM, Thompson EA, Perez EA. (2011). MicroRNA signatures: clinical biomarkers for the diagnosis and treatment of breast cancer. *Trends Mol Med* 17:313–319.

Baffa R, Fassan M, Volinia S, O'Hara B, Liu CG, Palazzo JP, Gardiman M, Rugge M, Gomella LG, Croce CM, Rosenberg A. (2009). MicroRNA expression profiling of human metastatic cancers identifies cancer gene targets. *J Pathol* 219:214–221.

Blenkiron C, Goldstein LD, Thorne NP, Spiteri I, Chin SF, Dunning MJ, Barbosa-Morais NL, Teschendorff AE, Green AR, Ellis IO, Tavaré S, Caldas C, Miska EA. (2007). MicroRNA expression profiling of human breast cancer identifies new markers of tumor subtype. *Genome Biol* 8:R214.

Bonnomet A, Syne L, Brysse A, Feyereisen E, Thompson EW, Noël A, Foidart JM, Birembaut P, Polette M, Gilles C. (2011). A dynamic *in vivo* model of epithelial-to-mesenchymal transitions in circulating tumor cells and metastases of breast cancer. *Oncogene*. [Epub ahead of print]

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 55:611–622.

Calin GA, Croce CM. (2006). MicroRNA signatures in human cancers. *Nat Rev Cancer* 6:857–866.

Eccles SA, Welch DR. (2007). Metastasis: recent discoveries and novel treatment strategies. *Lancet* 369:1742–1757.

Esquela-Kerscher A, Slack FJ. (2006). Oncomirs - microRNAs with a role in cancer. *Nat Rev Cancer* 6:259–269.

Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G, Vadas MA, Khew-Goodall Y, Goodall GJ. (2008). The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat Cell Biol* 10:593–601.

Heneghan HM, Miller N, Kerin MJ. (2011). Circulating microRNAs: promising breast cancer Biomarkers. *Breast Cancer Res* 13:402; author reply 403.

Heneghan HM, Miller N, Lowery AJ, Sweeney KJ, Newell J, Kerin MJ. (2010). Circulating microRNAs as novel minimally invasive biomarkers for breast cancer. *Ann Surg* 251:499–505.

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

Jackson RJ, Standart N. (2007). How do microRNAs regulate gene expression? *Sci STKE* 2007:re1.

Janssen EA, Slewa A, Gudlaugsson E, Jonsdottir K, Skaland I, Søiland H, Baak JP. (2010). Biologic profiling of lymph node negative breast cancers by means of microRNA expression. *Mod Pathol* 23:1567–1576.

Johnson ML, Navanukraw C, Grazul-Bilska AT, Reynolds LP, Redmer DA. (2003). Heparinase treatment of RNA before quantitative real-time RT-PCR. *BioTechniques* 35:1140–2, 1144.

Lowery AJ, Miller N, Devaney A, McNeill RE, Davoren PA, Lemetre C, Benes V, Schmidt S, Blake J, Ball G, Kerin MJ. (2009). MicroRNA signatures predict oestrogen receptor, progesterone receptor and HER2/neu receptor status in breast cancer. *Breast Cancer Res* 11:R27.

Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR. (2005). MicroRNA expression profiles classify human cancers. *Nature* 435:834–838.

Ma L, Teruya-Feldstein J, Weinberg RA. (2007). Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. *Nature* 449:682–688.

Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A, Lin DW, Urban N, Drescher CW, Knudsen BS, Stirewalt DL, Gentleman

R, Vessella RL, Nelson PS, Martin DB, Tewari M. (2008). Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci USA* 105:10513–10518.

Mostert B, Sieuwerts AM, Martens JW, Sleijfer S. (2011). Diagnostic applications of cell-free and circulating tumor cell-associated miRNAs in cancer patients. *Expert Rev Mol Diagn* 11:259–275.

Roth C, Rack B, Müller V, Janni W, Pantel K, Schwarzenbach H. (2010). Circulating microRNAs as blood-based markers for patients with primary and metastatic breast cancer. *Breast Cancer Res* 12:R90.

Shammas FV, Van Eekelen JA, Wee L, Heikkilä R, Osland A. (1999). Sensitive and quantitative one-step polymerase chain reaction using capillary electrophoresis and fluorescence detection for measuring cytokeratin 19 expression. *Scand J Clin Lab Invest* 59:635–642.

Shi M, Liu D, Duan H, Shen B, Guo N. (2010). Metastasis-related miRNAs, active players in breast cancer invasion, and metastasis. *Cancer Metastasis Rev* 29:785–799.

Tjensvoll K, Gilje B, Oltedal S, Shammas VF, Kvaløy JT, Heikkilä R, Nordgård O. (2009). A small subgroup of operable breast cancer patients with poor prognosis identified by quantitative real-time RT-PCR detection of mammaglobin A and trefoil factor 1 mRNA expression in bone marrow. *Breast Cancer Res Treat* 116:329–338.

Tjensvoll K, Oltedal S, Heikkilä R, Kvaløy JT, Gilje B, Reuben JM, Smaaland R, Nordgård O. Persistent tumor cells in bone marrow of non-metastatic breast cancer patients after primary surgery are associated with inferior outcome. *BMC Cancer*.

Velu CS, Baktula AM, Grimes HL. (2009). Gfi1 regulates miR-21 and miR-196b to control myelopoiesis. *Blood* 113:4720–4728.

Wong L, Lee K, Russell I, Chen C. (2007). Endogenous controls for real-time quantitation of miRNA using TaqMan MicroRNA assays. Application Note, Applied Biosystems.

Zhao H, Shen J, Medico L, Wang D, Ambrosone CB, Liu S. (2010). A pilot study of circulating miRNAs as potential biomarkers of early stage breast cancer. *PLoS ONE* 5:e13735.