A Serum MicroRNA Signature Predicts Tumor Relapse and Survival in Triple-Negative Breast Cancer Patients

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Abstract

Purpose: Triple-negative breast cancers (TNBC) are associated with high risk of early tumor recurrence and poor outcome. Common prognostic biomarkers give very restricted predictive information of tumor recurrences in TNBC. Human serum contains stably expressed microRNAs (miRNAs), which have been discovered to predict prognosis in patients with cancer. The purpose of this study was to identify circulating biomarkers able to predict clinical outcome in TNBC.

Experimental Design: We performed genome-wide serum miRNA expression and real-time PCR analyses to investigate the ability of miRNAs in predicting tumor relapse in serum samples from 60 primary TNBC. Patients were divided into training and testing cohorts.

Results: By Cox regression analysis, we identified a four-miRNA signature (miR-18b, miR-103, miR-107, and miR-652) that predicted tumor relapse and overall survival. This miRNA signature was further validated in an independent cohort of 70 TNBC. A high-risk signature score was developed and significantly associated with tumor recurrence and reduced survival. Multivariate Cox regression models indicated that the risk score based on the four-miRNA signature was an independent prognostic classifier of patients with TNBC.

Conclusion: This signature may serve as a minimally invasive predictor of tumor relapse and overall survival for patients with TNBC. This prediction model may ultimately lead to better treatment options for patients with TNBC. Clin Cancer Res; 1–8.

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Introduction

Triple-negative breast cancer (TNBC) is defined by the absence of estrogen receptor (ER), progesterone receptor (PgR), and HER2 expression and it accounts for approximately 15% to 20% of all breast cancers. Patients with TNBC show a significant worse prognosis compared with other breast cancer subtypes (1). However, increasing evidence indicates that TNBC is a heterogeneous disease that encompasses several distinct entities with remarkably different molecular characteristics and clinical behavior (1–3).

Studies on small noncoding RNA have demonstrated that microRNAs (miRNAs) are important regulators of gene expression in the human genome, thereby controlling a wide range of important biologic processes (5, 6). Recent evidences indicate that miRNAs may function as oncogenes or tumor suppressor genes, thus regulating key functions during tumor formation and progression (5). Several miRNAs have been shown to be stable and easily detectable in serum or plasma of patients with cancer and associated with clinicopathologic variables and clinical outcome (7–10). In this study, we investigated the prognostic role of the human miRNOME in serum samples derived from patients with primary TNBC. On the basis of this, we developed a miRNA signature significantly associated with early recurrences and poor outcome in patients with primary TNBC.
**Translational Relevance**

This study identifies circulating prognostic biomarkers for triple-negative breast cancers (TNBC). We demonstrated that a four-miRNA signature (miR-18b, miR-103, miR-107, and miR-652) may assist to accurately predict tumor relapse and overall survival in patients with TNBC. We developed a miRNA risk-scoring system able to assign patients to a high- or low-risk of tumor progression. This gene signature represents the first useful non-invasive prognostic biomarker for patients with TNBC, providing a comprehensive snapshot of changes occurring in response to tumor progression. Determining an accurate prediction of poor prognosis for TNBC is critical for the clinician to tailor the treatment plan for maximal efficacy and for the stratification of patients who may benefit the most from experimental clinical trials.

**Methods**

**Patients characteristic and serum specimens’ selection**

Primary ductal TNBCs were selected upon histological examination for this study. All the other histologic subtypes of TNBC tumors, in addition to rare forms of TNBC having a substantially less aggressive phenotype (apocrine, medullary, and squamous carcinoma) were excluded from the study.

All serum samples were collected from breast cancer patients before surgery and any therapy. Ethical approval was obtained from the review boards at the Norwegian, German, and Italian Institutions. All samples were derived from Caucasian patients.

Patients who developed distant metastases within 36 months after removal of the primary tumor were considered positive for tumor relapse, whereas patients who remained free of distant metastasis for the same time frame were defined as non-relapsing tumors.

In the discovery stage, we used serum samples from patients with TNBC comprising relapsing (n = 10) and non-relapsing (n = 10) tumors. To validate the significant identified circulating biomarkers and to develop a predictive risk score, a testing (n = 40) and an additional independent (n = 70) cohorts of serum samples were used. To consolidate our findings, a further analysis combining the two validation datasets (n = 110) was performed. All clinicopathologic characteristics are reported in Supplementary Table S1. In the analysis, we also looked at the prognostic impact of miRNAs in terms of overall survival (OS) in patients with TNBC having a minimum follow-up of 60 months.

After informed consent, 5 mL of venous blood was collected from each patient and processed within 1 hour. Serum samples were also collected from healthy women (n = 30) with no history of malignant diseases and from women with histologically confirmed ER-positive breast cancer (n = 33), using the same collection and sampling procedures.

**RNA isolation**

Total RNA was extracted from 250 μL of serum using the Exiqon’s miRCURY RNA isolation kit Biofluids (Exiqon), following the manufacturer’s guidelines and Exiqon’s specific application instructions. Briefly, a Qiazol mixture containing the MS2 bacteriophage RNA (Roche) was added to serum samples before RNA purification. Total RNA was eluted in RNase-free water and stored at −80°C until use.

**Quantitative real-time PCR analysis and miRNA expression**

Expression of miRNAs from serum samples included in the training cohort was evaluated using Exiqon miRCURY LNA Universal RT microRNA PCR (Exiqon). All miRNAs were polyadenylated and reverse transcribed into cDNA in a single reaction step. Each miRNA was assayed by quantitative real-time PCR (qRT-PCR) on the microRNA Ready-to-Use PCR, Human panel I and panel II v2 containing 742 miRNAs (Exiqon). Haemolysed samples were excluded by analyzing miR-451a expression levels. Negative controls without template were performed. Amplification was performed in a Roche Lightcycler 480 (Roche). MiRNAs expressed in all samples (n = 36) were used for normalization of miRNA expression. All data were normalized to the average of assays detected in all samples (average Ct - assay Ct).

Nine miRNAs, which were differentially expressed between relapsing and non-relapsing groups of patients in the training cohort (miR-18b-5p, miR-20a-5p, miR-30d-5p, miR-32-5p, miR-101-3p, miR-103a-3p, miR-107, miR-223-3p and miR-652-3p) were further analyzed in a testing (n = 40), and in an additional independent larger set of samples (n = 70). Briefly, a fixed volume from each sample, containing at least 20 ng of total RNA, were reverse transcribed using the TaqMan MicroRNA Reverse Transcription kit (Life Technologies). Each amplification reaction was performed in triplicate. MiRNA expression was quantified in relation to the expression of small nuclear U6 RNA. The relative expression of each miRNA was calculated with the 2^{–ΔΔCt} method.

**Statistical analysis**

In the discovery stage, the differential expression of miRNAs between patient subgroups was analyzed by unpaired two-tailed t test. For these analyses, we only considered miRNAs expressed in at least 75% of the samples. In the validation stage, P values were adjusted for multiple testing by Bonferroni correction. The Kruskal–Wallis rank test followed by a Dunn’s multiple correction test were used to determine the significance of miRNA expression between multiple subgroups. Hierarchical clustering was performed with Pearson correlation and average linkage using GENE-E software.

The associations between relapse-free survival (RFS) or OS and circulating miRNA expression levels were estimated by the Kaplan–Meier method, log-rank test (Mantel–Cox), and Cox proportional hazard regression models. To investigate the effectiveness of the miRNA signature for clinical outcome prediction, we assigned each patient a risk score according to a linear combination of the expression level of the miRNAs. The risk score function (RSF) for sample i using the information from the significant miRNAs was calculated as follows: $RSF_i = \sum_{j=1}^{n} W_j * s_{ij}$. In the above equation, $s_{ij}$ is the risk score for miRNA j on sample i, and $W_j$ is the weight of the risk score of miRNA j. Weights were obtained by the coefficients derived from the univariate Cox regression analyses as previously described (11). We divided patients into high-risk and low-risk groups using the median miRNA signature risk score as the cutoff point.

To evaluate the prognostic potential of individual miRNAs and sets of miRNAs, we generated receiver operating
characteristic (ROC) curves, and areas under the curves (AUC) were calculated. All tests were two-sided and the level of statistical significance was set at \( P < 0.05 \). Statistical analyses were performed using GraphPad Prism version 5, Epi Info version 7, and R software version 2.14.1.

**Results**

Expression of miRNAs in serum from patients with triple-negative breast tumors

After data filtering and normalization, we first determined the number of miRNAs detected in serum from patients with TNBC in the recurrent group and in the non-recurrent group in the training set. We found that the number of detectable miRNAs varied slightly between samples, allowing the detection of an average of 110 miRNAs expressed in each sample.

To make our analysis robust, we selected miRNAs expressed in at least 75% of the samples among the 742 human miRNAs analyzed in the training set. As a result, 71 serum miRNAs were identified and subjected to additional analyses (Supplementary Table S2). To identify differentially expressed miRNAs, we performed a two-sided \( t \)-test and generated a list of 9 significantly dysregulated miRNAs \( (P < 0.05) \) between recurrent and non-recurrent TNBC groups (Table 1). Two of these miRNAs (miR-32 and miR-101) were highly expressed in the non-relapsing group, and 7 miRNAs (miR-18b, miR-20a, miR-30d, miR-103, miR-107, miR-223, and miR-652) were highly expressed in the relapsing group (Supplementary Fig. S1). On the basis of the expression levels of these 9 circulating miRNAs, the 20 TNBC samples clustered into two groups (Supplementary Fig. S2).

The expression of this specific subset of miRNAs was then validated in a testing cohort of 40 TNBC samples, which comprised 20 patients who experienced tumor recurrence and 20 patients with no recurrence events. Among the 9 miRNAs analyzed, only 4 miRNAs (miR-18b, miR-103, miR-107, and miR-652) retained their prognostic significance \( (P < 0.05; \text{Bonferroni-adjusted}) \) in this cohort of patients (Table 1 and Fig. 1A).

Specificity for the expression of these miRNAs for TNBC was also evaluated by examining the serum samples from healthy women \( (n = 30) \) and from women with histologically confirmed ER-positive breast cancer \( (n = 33) \). All miRNAs showed consistently high levels in TNBC sera \( (P < 0.05) \) compared with healthy and ER-positive samples (Supplementary Fig. S3). No significant differences in the expression levels of these miRNAs were found between healthy and ER-positive subjects (Supplementary Fig. S3).

**Table 1. MicroRNA expression levels in recurrent triple-negative breast cancers**

<table>
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<tr>
<th>miRNA</th>
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<th>( P )</th>
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<th>( P )</th>
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**Note:** Significant \( P \)-values are shown in bold. Abbreviation: FC, fold change.

\*Two-sided \( t \)-test.

\*Bonferroni-adjusted.

Association between expression of the four circulating miRNAs and patient survival

The association between the circulating miRNAs expression and RFS or OS in patients with TNBC was evaluated by Kaplan–Meier and Cox proportional hazard regression analyses (Fig. 1B and C and Table 2). Patients in the testing set were divided into two groups based on median expression levels of each miRNA. Higher circulating levels of all the four miRNAs were individually associated with an increased risk of recurrence (Fig. 1B and Table 2), and decreased OS (Fig. 1C and Table 2).

We also considered the expression levels of the four miRNAs together. In the combined analysis, we found that patients with high expression of two or more miRNAs had significantly decreased RFS than patients carrying zero or one high-risk miRNA in the testing cohort \( (\log\text{-rank } P = 0.027; \text{HR} = 4.76; 95\% \text{CI}, 1.20–18.88 \text{ for } 2–3 \text{ high-risk miRNAs carriers}; \log\text{-rank } P = 0.001; \text{HR} = 6.80; 95\% \text{CI}, 2.16–21.39 \text{ for } 4 \text{ high-risk miRNAs carriers}; \text{Fig. 1B}) \). Similarly, a consistent decrease in OS was found in patients with high expression of two or more miRNAs compared with patients carrying zero or one high-risk miRNA in the testing cohort \( (\log\text{-rank } P = 0.008; \text{HR} = 7.25; 95\% \text{CI}, 1.69–31.18 \text{ for } 2–3 \text{ high-risk miRNAs carriers}; \log\text{-rank } P = 0.0005; \text{HR} = 7.78; 95\% \text{CI}, 2.43–24.91 \text{ for } 4 \text{ high-risk miRNAs carriers}; \text{Fig. 1C}) \).

We then used the four miRNAs to construct a signature by a risk score method. The patients in the testing set were ranked according to their risk scores and divided into high-risk or low-risk groups using the median risk score as the cutoff point. Patients with a high-risk four-miRNA signature had significantly shorter RFS than patients with low-risk miRNA signature \( (\log\text{-rank } P = 0.002; \text{Fig. 2A}) \). Likewise, the four-miRNA signature was consistently associated with a reduced OS \( (\log\text{-rank } P = 0.0007; \text{Fig. 2A}) \).

To confirm that the set of four miRNAs is essential for the prognostic value of the signature, we constructed four alternative miRNA signatures, each composed by 3 miRNAs, by deleting one miRNA in turn from the original four-miRNA signature. We then repeated the Kaplan–Meier survival analysis for each of these alternative signatures and compared the results with the original one using the log-rank analysis. The results showed that the four-miRNA signature exhibited significantly greater performance than other alternative signatures in predicting both RFS and OS in the testing cohort of patients with TNBC (data not shown).

To validate the prognostic role of this four-miRNA signature, we applied the same risk-score formula and cut-off point obtained from the testing cohort to additional 70 TNBC patients from an independent cohort and to a combined dataset, which included the two cohorts of samples \( (n = 110; \text{Fig. 2}) \). In both analysis we confirmed that patients with a high-risk miRNA signature had a significant shorter RFS than patients with a low-risk miRNA signature \( (\log\text{-rank } P = 0.0005 \text{ for the independent set}; \log\text{-rank } P < 0.0001 \text{ for the all-combined 110 patients}; \text{Fig. 2B and C}) \). Likewise, patients with a high-risk four-miRNA signature had significantly reduced OS compared with patients with low-risk miRNA signature \( (\log\text{-rank } P = 0.0003 \text{ for the independent set}; \log\text{-rank } P < 0.0001 \text{ for the all-combined 110 patients}; \text{Fig. 2B and C}) \). Furthermore, univariate and multivariate Cox regression analysis, adjusted for age at diagnosis, tumor size, lymph node status, tumor stage and histological grade, showed that the miRNA signature was significantly associated with reduced RFS and OS in both cohorts (Table 2 and Supplementary Table S3). In the multivariate analysis, the four-miRNA signature, despite a
borderline statistical significance of lymph node status, retained its strong independent prognostic significance (Supplementary Table S3). We also showed the distribution of circulating miRNA expression levels, patient risk scores, and the OS status of the combined 110 patients (Fig. 3). Patients with high-risk score had the tendency to express high levels of the four miRNAs and to have shorter OS than patients with low-risk score.

The prognostic accuracy of single miRNAs as well as four-miRNA signature to distinguish between recurrent and non-recurrent TNBCs (n = 110) was assessed by a ROC test (Fig. 4). Considered individually, miR-18b, miR-103, miR-107, and miR-652 showed significant high AUC scores (0.701, 0.712, 0.742, and 0.752, respectively). However, the four-miRNA signature showed the strongest predictive value to discriminate tumors from patients with early relapse from those without recurrence, with an AUC = 0.810 (Fig. 4), demonstrating that the miRNA signature has a good performance as a risk predictor for early breast cancer recurrence.
In this study, we demonstrated for the first time that circulating miRNAs measured at the time of diagnosis can be used to predict disease progression in primary TNBC. Quantification of circulating miRNAs has only recently become feasible and reliable, with most efforts focusing on miRNAs dysregulated in specific cancer cells. Our results demonstrated that the expression pattern of four miRNAs (miR-18b, miR-103, miR-107 and miR-652) was consistently altered in TNBC patients with poor outcome. Our data clearly indicate that a single specific circulating miRNA may assist to predict the clinical outcome of patients with TNBC, although only the analyses of the combined four miRNAs had the greatest performance to predict both RFS and OS. Accordingly, patients

<table>
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<th>Variable</th>
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<th>P</th>
<th>Multivariate HR (95% CI)</th>
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<td>Testing cohort</td>
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NOTE: Multivariate analysis adjusted for histological grade (1–2/3), age (median), nodal status (0/1), tumor size (median), and stage (I–II/III). Significant P-values are shown in bold.

Abbreviations: HR, hazard ratio; CI, confidence interval.

Discussion

In this study, we demonstrated for the first time that circulating miRNAs measured at the time of diagnosis can be used to predict disease progression in primary TNBC. Quantification of circulating miRNAs has only recently become feasible and reliable, with most efforts focusing on miRNAs dysregulated in specific cancer cells. Our results demonstrated that the expression pattern of four miRNAs (miR-18b, miR-103, miR-107 and miR-652) was consistently altered in TNBC patients with poor outcome. Our data clearly indicate that a single specific circulating miRNA may assist to predict the clinical outcome of patients with TNBC, although only the analyses of the combined four miRNAs had the greatest performance to predict both RFS and OS. Accordingly, patients
predictive value (AUC). When miRNAs have a high AUC score, the miRNA signature has the strongest ability to discriminate between patients with recurrence and those without recurrence in the combined testing and independent cohorts. Although single miRNAs have a high AUC score, the miRNA signature has the strongest predictive value (AUC = 0.810) to discriminate those patients.

Figure 3.
miRNA risk score analysis of 110 patients with TNBC. A, miRNA risk score distribution. B, patients’ OS. C, color-ga plot of miRNA expression profiles of patients with TNBC; rows, miRNAs; columns, patients. The dotted line represents the median miRNA signature cutoff dividing patients into low-risk and high-risk groups.

with the concurrent presence of all four miRNA in the sera had a very high likelihood of aggressive disease behavior. Prognostic markers indicative of poor outcome in TNBC have been elusive so far, and some of them are not particularly well categorized. In addition, preoperative clinical parameters derived by tumor biopsy can be affected by undersampling and molecular heterogeneity. Determining an accurate prediction of prognosis at the time of diagnosis is critical for clinicians to tailor the treatment plan for maximal efficacy and to determine surveillance strategies. In this respect, circulating miRNAs can be measured constantly and non-invasively, potentially allowing for a continuous monitoring and assisting in treatment decision making (12).

Despite the biologic significance of this subset of four miRNAs in the blood of patients with aggressive TNBC needs to be established, these miRNAs have already been associated with metastatic features and chemotherapy resistance in tumor tissues. The nature of these four miRNAs and related targets is suggestive of their functions being regulators of various properties of cancer cells, such as cancer stem cells, epithelial to mesenchymal transition (EMT), genomic stability, and immune response, which are all well recognized processes of tumor progression (13–16). Our study suggests that some of these functions in TNBC may be regulated by this specific subset of miRNAs. miR-103 and miR-107 have earlier been implicated in EMT and DNA repair, and associated with metastasis and poor outcome in breast cancer (17–19). miR-652 has been demonstrated to identify stage I–II breast cancer patients and to correlate with TP53, suggesting a potential role for this miRNA as prognostic biomarker (20). While the biological role of miR-652 has not been clarified in breast cancer yet, there is evidence that demonstrates the oncogenic properties of this miRNA in other cancer types (21–23). Finally, ER has been found to be a direct target of miR-18b. Overexpression of miR-18b has been shown to be associated with basal-like/TNBC and TP53 mutation status, and to correlate with worse survival in HER2-negative breast cancer (24–26).

Some limitations may apply to this study. Although the potential contamination of serum miRNAs derived from blood cells was eliminated, relative boundaries of this research may be linked to the mechanisms that control the generation of circulating miRNAs. The underlying mechanisms involved in the release of miRNAs into the blood stream of patients with cancer could be diverse (27). Despite several studies have shown the intercellular transfer of miRNAs from donor cells to recipient cells, only few reports have clearly demonstrated the mechanism of secretion of miRNAs (e.g., microvesicles, exosomes, circulating tumor cells, and apoptotic bodies can transfer miRNAs between the cells; ref. 28). Although the mechanisms of secretion and incorporation of miRNAs have not been well clarified, secretory miRNAs seem to play a pivotal role as signaling molecules in cancer. In fact, the biologic mechanisms of breast cancer progression, and particularly of distant recurrence, may be driven by the primary tumor’s genomic features, but also by complex systemic signals potentially regulated by circulating miRNAs (e.g., modulation of the local tumor microenvironment or the immune system response). Overall, circulating miRNAs are likely to provide a comprehensive snapshot of changes occurring in response to tumor growth and disease progression, thus overcoming potential issues related to tumor sampling and molecular heterogeneity.

Despite these potential limitations correlated with the use of such liquid biomarkers, it is worth reinforcing the utility of circulating tumor miRNAs that may assist in a rapid assessment of prognosis in TNBC, a group that engenders considerable clinical uncertainty. Even though other prognostic gene signatures have been developed from TNBC tissues (29–32), our study is the first to identify a specific miRNA signature in the serum of patients with TNBC correlated with the high likelihood of tumor relapse. Furthermore, despite their molecular heterogeneity, all newly diagnosed basal-like/TNBC cases are treated with standard adjuvant combination chemotherapy, although with unsatisfactory results. Moreover, there is not a proven effective single agent that
targets a driving vulnerability in TNBC. Our data indirectly suggest that the group of patients with high expression of the miRNA signature is probably inadequately treated with the currently available chemotherapy. As a result, testing the expression levels of these serum biomarkers will provide the opportunity to identify subgroups of patients who should be included in novel randomized clinical trials for alternative treatment strategies.

This study, if confirmed in prospective clinical trials, indicates that this miRNA signature could be a significant non-invasive tool to determine patients at high risk of tumor recurrence and be the first to stratify this class of tumors based on their potential metastatic behavior, thus offering timely treatment alternatives.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: K. Kleivi Sahliberg, A.-L. Børresen-Dale, L. Santarpia
Development of methodology: G. Bottai, B. Burwinkel, L. Santarpia
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. Bottai, B. Naume, B. Burwinkel, A.-L. Børresen-Dale, L. Santarpia
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Kleivi Sahliberg, G. Bottai, A-L. Børresen-Dale, L. Santarpia

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