Association of miR-548c-5p, miR-7-5p, miR-210-3p, miR-128-3p with recurrence in systemically untreated breast cancer

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ABSTRACT

Current prognostic markers allocate the majority of lymph node (LN) negative and estrogen receptor (ER) positive breast cancer patients into the high-risk group. Accordingly, most patients receive systemic treatments although approximately 40% of these patients may have been cured by surgery and radiotherapy alone. Two studies identified seven prognostic microRNAs in systemically untreated, LN negative and ER positive breast cancer patients which may allow more precise patient classification. However, six of the seven microRNAs were analyzed in both studies but only found to be prognostic in one study. To validate their prognostic potential, we analyzed microRNA expression in an independent cohort (n = 110) using a pair-matched study design minimizing dependence of classical markers. The expression of hsa-miR-548c-5p was significantly associated with abridged disease-free survival (hazard ratio [HR]:1.96, \( p = 0.027 \)). Contradicting published results, high hsa-miR-516-3p expression was associated with favorable outcome (HR:0.29, \( p = 0.0068 \)). The association is probably time-dependent indicating later relapse. Additionally, re-analysis of previously published expression data of two matching cohorts (n = 100, n = 255) supports an association of hsa-miR-128-3p with shortened disease-free survival (HR:2.48, \( p = 0.0033 \)) and an upregulation of miR-7-5p (\( p = 0.0038; \ p = 0.039 \)) and miR-210-3p (\( p = 0.031 \)) in primary tumors of patients who experienced metastases. Further analysis may verify the prognostic potential of these microRNAs.

INTRODUCTION

Breast cancer is the most frequent cancer in women worldwide and is associated with a high mortality rate of approximately 15% despite recent improvements in diagnosis and treatment [1, 2]. The main cause of death is not the primary tumor in the breast, but the occurrence of lethal metastases in essential organs like lung, liver and the brain. Lymph node (LN) negative breast cancer patients do not present any signs of distant metastasis at the time of diagnosis. However, clinical and histopathological criteria currently classify the majority of LN negative and estrogen receptor (ER) positive patients as high-risk patients who accordingly receive adjuvant systemic treatments after surgical removal of the primary tumor [2]. In fact, approximately 40% of these patients do not benefit from the systemic treatment and would be cured by the removal of the primary tumor and radiotherapy.
In the past decades microRNAs (miRs) have intensively been studied in primary breast tumor tissue and a few prognostic miRs have been proposed [8–10]. Although miRs are considered as key-regulators of the breast cancer transcriptome, none of the so far identified prognostic miRs have, to our knowledge, passed larger clinical trials [11]. Bias concerning the analyzed sample material, patient cohort heterogeneity as well as methodological and analytical variation limits the initial validation of candidate miRs. Lánczky et al. recently succeeded in validating the predictive potential of 26 out of 41 selected miRs by analyzing e.g. subtype- or receptor-specific patient cohorts with the integrated platform miRPower in a large group of mainly systemically treated patients [12]. In comparison, two studies focused solely on the de novo identification of prognostic miRs in primary tumor samples of LN negative, ER positive patients who did not receive any systemic treatment [7]. However, better prognostic markers are required to substantially increase the ultralow patient group and to ensure that the rate of recurrence will not increase as a consequence of treatment reduction.

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RESULTS

D’Aiuto et al. identified hsa-miR-1308, hsa-miR-548c-5p (MIMAT0004806), hsa-miR-125b-5p (MIMAT000423) and hsa-miR-30e-3p as differentially expressed (p < 0.001) in LN negative, ER positive and ERBB2 negative breast cancer patients [14, 19]. The authors confirmed the prognostic role of hsa-miR-30e-3p in a matching cohort of 223 untreated patients extracted from the Metabric cohort [15, 16]. We aimed at validating the candidate miRs (except for hsa-miR-1308 which was later identified as being part of a t-RNA) [19] in our independent LN negative, ER positive cohort using a paired study design. To match our patient characteristics and analysis parameters, we further re-analyzed the role of these miRs in the D’Aiuto et al. and Metabric cohorts (Table 1) but included ERBB2 positive samples and divided the samples into high/low expression groups as being above or below the median expression value.

While we could not confirm a significant expression difference of hsa-miR-125b-5p or hsa-miR-30e-3p (Figure 1A and 1B, Table 2) we observed a trend showing a moderate upregulation of miR-30e-3p in primary tumors of patients who did not develop metastasis (p = 0.17). The same trend is also reflected by the survival curves considering disease-free and overall survival (Figure 1B and Table 2; hazard ratio [HR]:0.64, p = 0.31 and HR:0.82, p = 0.66 respectively). In comparison, a significant expression upregulation in patients with relapse could be demonstrated for hsa-miR-548c-5p...
Furthermore, high hsa-miR-548c-5p expression was associated with abridged disease-free survival (HR: 2.63, \( p = 0.020 \)) and tended to result in shorter overall survival (HR: 2.25, \( p = 0.056 \)) (Figure 1C, Table 2). Potential reasons may be a more heterogeneous cohort composition with shorter follow up, varying sample treatment or a possible cross-reactivity of the applied array format (customized Agilent array) [20] considering that hsa-miR-548c-5p belongs to a rather large miR family comprising 74 human miRs with similar
sequences [19]. Although the Metabric cohort comprises more patients, the statistical power is not fundamentally superior in comparison to the other cohorts because of the unbalanced distribution of patients with (n = 65) and without (n = 190) recurrence.

Foekens et al. identified four miRs in 37 LN negative, ER positive primary breast tumor samples and validated their association with reduced DFS when highly expressed in further 147 LN negative, ER positive primary tumors [13]. We evaluated the differential expression of these miRs in our matching data set but could neither confirm a differential expression of hsa-miR-7-5p (MIMAT0000252), hsa-miR-128-3p (MIMAT0000424) and hsa-miR-210-3p (MIMAT0000267), nor a specific association with abridged disease-free or overall survival (Figure 2A, 2D, 2G; Table 3) [19]. Furthermore, opposing Foekens et al. results, we found hsa-miR-516-3p (hsa-miR-516a-3p; hsa-miR-516b-3p; MIMAT0002860; MIMAT0006778) [19] significantly downregulated in primary tumors of patients who developed later metastasis accompanied by a significant association of low hsa-miR-516-3p expression with metastasis formation (HR:0.29, p = 0.0068) and shorter overall survival (HR: 0.26, p = 0.0079; Figure 2J).

To further clarify the potential role of these four miR candidates, we additionally analyzed their expression and outcome association in the available D'Aiuto et al. and Metabric data sets (Figure 2; Table 3) [14–16]. A differential expression of hsa-miR-7-5p was found in the D'Aiuto et al. (p = 0.0038; Figure 2B) and the Metabric cohort (p = 0.039; Figure 2C) supporting Foekens et al. initial results. However, no significant association with disease free survival (DFS) or overall survival was detected. For hsa-miR-128-3p a prognostic role could be determined in the D'Aiuto et al. set (Figure 2E) where a high expression was significantly associated with poor outcome (HR:2.48, p = 0.0033). No significant association was computed for hsa-miR-128-3p in the Metabric cohort (Figure 2F). Foekens et al. demonstrated a prognostic role for hsa-miR-210-3p in ER positive and negative breast cancer, while the latter was not in the scope of this study, a significant correlation between outcome and hsa-miR-210-3p expression in ER positive patients could not be validated (Figure 2G, 2H, 2I; Table 3) although expression was significantly upregulated in samples from patients with relapse in the D'Aiuto et al. data set (Figure 2H). Hsa-miR-516-3p was not analyzed in the Metabric study and data available for the D'Aiuto et al. cohort did not

Figure 1: Differential expression and association with outcome for hsa-miR-125b-5p (A), hsa-miR-30e-3p (B) and hsa-miR-548c-5p (C). Boxplots present differences in expression patterns between patients who developed later metastasis (MET) and those who did not (NON-MET). Whiskers correspond to the upper and lower 25% range, data points deviating more than 3*STDEV from the mean are displayed as dots. Lighter colors indicate the 50–75 percentile range, while darker colors indicate the 25–50 percentile range of miRs expressed in MET (red) and NON-MET (blue). Significance was calculated using a paired Student’s T-test for the OUH cohort (left panel) and an unpaired Student’s T-test for the D’Aiuto et al. and Metabric cohort. Kaplan Meier plots indicating disease free (DFS) and overall survival (OS) probabilities were constructed using a univariable COX regression model. Results from the OUH cohort (n = 110) are displayed in line with re-analyzed results for the D’Aiuto et al. (n = 100) and the Metabric (n = 255) cohort (encased in grey) as published previously [14].
confirm an advantageous low or high expression although a trend towards a beneficial low expression could be observed (HR: 0.61, p = 0.090; Figure 2K, Table 3). Methodological differences and variable cohort features could explain the bias between Foekens et al. and our results. For example, in comparison to the D’Aiuto et al. and the Foekens et al. data sets, we could grasp back to a cohort with an almost double so long follow-up period including patients with late recurrence 5 years after the initial diagnosis (Table 1). Considering further the intersecting arms in the survival curves displayed in Figure 2J, we analyzed whether hsa-miR-516-3p expression may be time dependent using the Schoenfeld residual test [21]. The test revealed that the proportional hazards assumption was significantly violated with respect to DFS (p = 0.0012) and OS (p = 0.001) (Figure 3A and 3C), thus precluding the application of the COX regression model [21, 22]. To account for non-proportionality we conducted a landmark analysis using two time intervals (Figure 3B and 3D). The intervals were defined based on the time points at which the hazard ratio assessed by the Schoenfeld residuals changes from above one to below one. For DFS and OS an even more significant association of low hsa-miR-516-3p expression with an unfavorable prognosis was determined 3.35 (HR: 0.15, p = 0.0018) and 4.5 years (HR: 0.06, p = 0.00030) post diagnosis, respectively (Figure 3B and 3D). However, no significant association could be detected in the first time interval although a trend was observed pointing in the opposite direction. In summary, our results contradict the initial findings by Foekens et al. but may point to a time-dependent prognostic role of hsa-miR-516-3p.

**DISCUSSION**

A general challenge for the identification and validation of prognostic miR markers is the suspected low comparability between standalone studies. Potential prognostic candidates are individually identified dependent on the quality of RNA and clinical data, patient characteristics as well as the selected study design and methodology. Stringent statistical filtering and correction for multiple testing often limits the number of assigned positive hits. As a result, the majority of miRs are classified as negative hits although one cannot rule out that a number of promising candidates may have been filtered out. Other studies may identify these as promising

| Table 2: Overview summarizing expression and survival analysis for miRs identified by D’Aiuto et al. [14] |
|---------------------------------|---------------------------------|---------------------------------|
|                                | D’Aiuto et al.                  | Metabric*                        | OUH                            |
|                                | identification                 | 1st validation                   | 2nd validation                  |
| hsa-miR-125b-5p                |                                |                                 |                                |
| Diff. Exp.                     | p-value 0.0015                 | down in MET                      |                                |
|                                | p-value 0.018                  |                                  |                                |
| DFS                            | HR (CI) 0.47 (0.21–0.75)       | -                               | 0.92 (0.42–2.02)               |
|                                | p-value 0.081                  |                                  | 1.00                            |
| OS                             | HR (CI) -                      | 0.64 (0.39–1.06)                 | 1.00 (0.43–2.31)               |
| hsa-miR-30e-3p                 |                                |                                 |                                |
| Diff. Exp.                     | p-value 0.0005                 | down in MET                      |                                |
|                                | p-value 0.0094                 |                                  |                                |
| DFS                            | HR (CI) 0.45 (0.25–0.82)       | -                               | 0.64 (0.28–1.49)               |
|                                | p-value -                      | 0.028                           | 0.66                            |
| OS                             | HR (CI) -                      | 0.57 (0.34–0.94)                 | 0.82 (0.34–1.97)               |
| hsa-miR-548c-5p                |                                |                                 |                                |
| Diff. Exp.                     | p-value 0.010                  | up in MET                        | up in MET                       |
|                                | p-value 0.027                  |                                  | 0.012                           |
| DFS                            | HR (CI) 1.96 (1.08–3.56)       | -                               | 2.63 (1.16–5.93)               |
|                                | p-value -                      | 0.36                            | 0.056                           |
| OS                             | HR (CI) -                      | 1.26 (0.77–2.06)                 | 2.25 (0.98–5.18)               |

*re-evaluation of already published results [14]; Diff. Exp.: Differential expression; MET: primary tumors from patients with relapse; HR: Hazard ratio; CI: 95% Confidence interval.
candidates such that different standalone studies rarely identify overlapping candidate sets [12]. Although, subsequent validation in independent cohorts is often applied to confirm the prognostic potential of a positive candidate, unfortunately, the confirmation rate is likely to be low because of the aforementioned heterogeneity between studies. However, the successful reproduction of results for a positive hit is indeed expected to have increased explanatory power, because it is very unlikely that the same biomarker shows significant results in two truly independent cohorts just by chance.

We evaluated the prognostic potential of seven previously proposed miRs in systemically untreated breast cancer patients independent of classical clinical and pathological marker. Besides analyzing publicly available data sets we introduce our new data set comprising 110 paired primary tumors of systemically untreated patients with very long follow up time. Matching of the samples minimized bias concerning clinical and pathological characteristics as well as bias related to storage time, sampling method, and diagnostic procedures. Consequently, the chosen paired design increased the power of our analysis in comparison to cohort studies by omitting any effect potentially caused by an unbalanced cohort composition and by enriching for relevant clinical endpoints [18].

We found an association of hsa-miR-548c-5p with outcome in our data set supporting previously published results. For the remaining candidate miRs no significant validation results were obtained. A lack of power due to the lower number of samples analyzed in our study may cause the bias. However, the use of untreated samples with prolonged follow up was a prerequisite to search for potential marker which can identify patients who may not benefit from any systemic treatment.

In comparison, the re-analysis of published data supported a prognostic role of hsa-miR-30e-3p, hsa-miR-7-5p, hsa-miR-210-3p and hsa-miR-128-3p. However, none of the analyzed candidate miRs could be confirmed as being prognostic in all four tested cohorts. For two miRs, significant results were only computed in a single cohort. Although we aimed at finding best matching

Figure 2: Differential expression of hsa-miR-7-5p, hsa-miR-128-3p, hsa-miR-210-3p and hsa-miR-516-3p. Boxplots present differences in expression patterns between patients who developed later metastasis (MET) and those who did not (NON-MET) from the OUH, the D’Aiuto et al. and the Metabric cohort (panels from left to right). Whiskers correspond to the upper and lower 25% range, data points deviating more than 3*STDEV from the mean are displayed as dots. Lighter colors indicate the 50–75 percentile range while darker colors indicate the 25–50 percentile range of miRs expressed in MET (red) and NON-MET (blue). Significance was calculated using a paired Student’s T-test for the OUH cohort (A, D, G, J) and an unpaired Student’s T-test for the D’Aiuto et al. (B, E, H, K) and Metabric cohort (C, F, I). Kaplan Meier plots display disease free (DFS) and overall survival (OS) along with the univariate COX-PH regression significance levels.
cohorts, we were limited to systemically untreated samples of LN negative and ER positive patients. Moreover, the comparability of the included cohorts may not be optimal considering e.g. that only marginal clinical data were available for the Foekens et al. cohort or, that the Metabric cohort was quite heterogeneous. Furthermore, in all previously published studies considerable shorter follow-up times were available. These differences as well as variances in sample handling and differences in the miR purification protocol may have reduced the reproducibility across different studies. Result bias may further be caused by varying platform performance considering reproducibility, accuracy, detection rate, sensitivity and specificity [20, 23, 24]. Foekens et al. quantified miRs using a qPCR-based approach (TaqMan, Applied Biosystems) while D’Aiuto et al. used MicroRNA expression beadchips from Illumina. Interestingly, results for three out of four miRs identified by Foekens et al. were validated using the D’Aiuto et al. data set potentially indicating an improved comparability between technologies including PCR amplification steps. In comparison, data collected based on miR hybridization on customized arrays produced by Agilent (Metabric) and on our own arrays based on a probe library provided by Exiqon verified results for three of the seven suggested candidates. Mestdagh et al. analyzed the differential expression of two identical sample groups via 12 platforms (applying qPCR, hybridization or sequencing

| Table 3: Overview summarizing expression and survival analysis for miRs identified by Foekens et al. [13] |
|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| hsa-miR-7-5p                                      | Foekens et al. identification | OUH validation | D’Aiuto et al. validation | Metabirc validation |
|-------------------------------------------------|--------------------------|----------------|------------------------|-------------------|
| Diff. Exp.                                      | p-value                  | up in MET     | 0.83                   | up in MET         | up in MET         |
|                                                   |                          |               |                        | 0.0038            | 0.039             |
| DFS                                             | p-value                  | ≤0.05         | 0.51                   | 0.082             | -                 |
| HR (CI)                                         | >1                       | 0.75 (0.32–1.78) | 1.68 (0.94–3.01) | 1.68 (0.94–3.01) | -                 |
| OS                                              | p-value                  | -             | 0.66                   | -                 | 0.41              |
| HR (CI)                                         | -                        | 0.82 (0.34–1.97) | -                     | 1.23 (0.75–2.02) |                   |
| hsa-miR-128-3p                                   | Diff. Exp.               | p-value       | up in MET              | 0.61              | up in MET         | 0.95 |
|                                                   |                          | ≤0.05         | 0.20                   | 0.008             | -                 |
| DFS                                             | HR (CI)                  | >1            | 1.64 (0.77–3.47)       | 2.48 (1.35–4.55)  | -                 |
| OS                                              | p-value                  | -             | 0.18                   | -                 | 0.31              |
| HR (CI)                                         | -                        | 1.70 (0.78–3.71) | -                     | 0.77 (0.47–1.27) |                   |
| hsa-miR-210-3p                                   | Diff. Exp.               | p-value       | up in MET              | 0.22              | up in MET         | 0.78 |
|                                                   |                          | ≤0.05         | 0.83                   | 0.070             | -                 |
| DFS                                             | HR (CI)                  | >1            | 0.91 (0.39–2.14)       | 1.71 (0.96–3.07)  | -                 |
| OS                                              | p-value                  | -             | 0.83                   | -                 | 0.15              |
| HR (CI)                                         | -                        | 0.91 (0.39–2.14) | -                     | 1.44 (0.88–2.35) |                   |
| hsa-miR-516-3p                                   | Diff. Exp.               | p-value       | up in MET              | down in MET       | 0.089             | - |
|                                                   |                          | ≤0.05         | 0.0068                 | 0.090             | -                 |
| DFS                                             | HR (CI)                  | >1            | 0.29 (0.12–0.71)       | 0.61 (0.34–1.08)  | -                 |
| OS                                              | p-value                  | -             | 0.0079                 | -                 | -                 |
| HR (CI)                                         | -                        | 0.26 (0.098–0.70) | -                     | -                 | -                 |

*according to published results [13]; Diff. Exp.: Differential expression; MET: primary tumors from patients with relapse; HR: Hazard ratio; CI: 95% Confidence interval.
for analysis) and detected 66 differentially expressed miRs by at least one platform [20]. However, only two of these miRs were concordantly detected as differentially expressed by all platforms [20]. Thus, platform-dependent false positive and negative hits are likely to occur and the careful selection of similar performing platforms may be critical for a robust validation of candidate miRs when using different cohorts.

Previous studies further attempted to causally link the seven miR candidates to specific functions in breast cancer progression. For example, D’Aiuto et al. reported that the prognostic hsa-miR-30e-3p is functionally linked to the induction of epithelial-mesenchymal-transition (EMT) which is a prerequisite progress for tumor cell dissemination [14]. This result is in line with previous studies suggesting a critical role of the miR-30-family in EMT [25, 26]. We found high hsa-miR-548c-5p expression significantly associated with abridged disease-free-survival. The prognostic role of this miR may even be independent of the receptor status or treatment, because Boukerroucha et al. identified a predictive role of hsa-miR-548c-5p in conjunction with three other clinic-histopathological parameters in triple negative breast cancer (TNBC) [27]. On the contrary, functional studies in endometrial, ovarian and liver cancer suggest that decreased hsa-miR-548c expression promotes cell migration and invasion potentially by silencing the EMT marker TWIST [28, 29]. Accordingly, further studies in ER positive breast cancers are required to verify the functional and prognostic role of these miRs.

In comparison, miR-7 was shown to function as breast cancer invasion and metastasis inhibitor and to mediate cytotoxic T-lymphocyte-mediated lysis of breast cancer cells [10, 30]. Moreover, miR-7 was shown to suppress cell proliferation and induce apoptosis in different breast cancer cell lines [31]. Conversely, Foekens et al. associated a high expression of hsa-miR-7-5p with abridged disease free survival and; we found hsa-miR-7-5p significantly upregulated in metastasizing tumors of the D’Aiuto et al. cohort. Differences in receptor status, strand selection and endogenous expression may compromise comparability of these studies and may explain varying functional roles for miR-7 dependent on the examined cellular background.

MiR-210 has also extensively been analyzed in breast cancer and several studies associated miR-210 expression

![Figure 3: Scaled Schoenfeld residuals for hsa-miR-516-3p and survival analysis using two time intervals.](image)

The scaled Schoenfeld residuals (A, C) are shown by circles. The black line is the smoothed mean of the scaled Schoenfeld residuals while the corresponding 95% confidence interval is indicated by the dashed lines. The blue horizontal line corresponds to a hazard ratio of one, and the red horizontal line is the hazard ratio assessed by the univariable COX proportional hazard ratio model (0.29 and 0.26 for DFS and OS, respectively; Figure 2J). The vertical green line shows the time point upon which the hazard ratio shifts from a value >1 to a value <1, representing the time point when the prediction association changes. These time points were used in a subsequent landmark analysis to define two time intervals for disease-free (B) and overall survival analysis (D) in which the proportional hazard assumption was fulfilled.
with breast cancer survival [9, 10, 12, 32]. However, we could not confirm the prognostic role of hsa-miR-210-3p in primary tumor samples although a significant differential expression was detected in local versus disseminating primary tumors [33]. Wang et al. performed a systematic meta-analysis of miR-210 in different cancer types and found no significant association with breast cancer [34]. Contradicting results were reported by Xie et al. and Hong et al. in two meta-analysis studies [35, 36]. However, all studies included mixed patient cohorts including treated patients and varying receptor status. The same is true for functional studies which linked miR-210 to treatment resistance, hypoxia and a cancer promoting role in ER negative breast cancers [10, 32].

Aberrant expression of miR-128 was reported in many different malignancies including e.g. breast, colon, lung, brain and pancreatic tumors [37, 38]. Buffa et al. significantly associated high hsa-miR-128a with reduced distant relapse free-survival in ER positive breast cancer samples receiving systemic adjuvant treatments [33]. They further confirmed their results via an integrative meta-analysis and Lánczky et al. independently validated the predictive role of miR-128a [12, 33]. These findings are in good agreement with our results. However, these studies included treated patients and Masri et al. associated high miR-128 expression with letrozole resistance in breast cancer [39]. Consequently, the results may suggest that miR-128a may promote breast cancer recurrence by not only promoting metastasis formation but also by mediating treatment resistances.

Surprisingly, hsa-miR-125b-5p was neither differentially expressed nor significantly associated with survival in our study, although a tumor suppressive role of miR-125b was recurrently reported in cell line models [40, 41]. Moreover, miR-125b is part of a 10-miR classifier which significantly predicts 5-year distant relapse free survival in endocrine treated, hormone receptor positive and ERBB2 negative breast cancer patients [42]. In summary, miR-125b-5p may positively support endocrine treatments but may not be causal for the occurrence of metastases.

In comparison to the other analyzed miRs, little is known about the functional role of miR-516a. White et al. reported, that miR-516a is one out of three miRs regulating kallikrein 10 expression and cell proliferation in breast cancer [43]. The database Tarbase (http://www.microrna.gr/tarbase) proposes sulfatase 1 (SULF1) as the only validated gene target of hsa-miR-516a-3p [44]. SULF1 was reported to be a tumor suppressor, and to be downregulated in primary breast cancer tissue [45]. Correspondingly, an upregulation of hsa-miR-516a-3p is assumed to reduce SULF1 and the positive effects mediated by the gene which is contradicting our findings, that a low hsa-miR-516a-3p is associated with unfavorable outcome. However, our findings oppose the initial outcome association published by Foekens et al. [13]. Unfortunately, no patient specific expression data and clinical and pathological information were available for the Foekens et al. cohort which would allow us to re-analyze the data and to potentially point out causal difference in the cohort composition which may explain contradicting results. While this miR was not included in the Metabric study, an analysis in the independent D’Aiuto et al. cohort did not significantly indicate a beneficial up- or downregulation of the miR, although we observed a slight trend towards an advantageous high expression. However, follow up of patients was much longer in our study. Furthermore, we had to divide our data into two time intervals to not violate the proportional hazards assumption of the COX model. We observed, that high expression is potentially associated with a worse prognosis within the first years after diagnosis (3.35 and 4.5 years for DFS and OS, respectively), while thereafter high expression is significantly associated with prolonged survival. It is acknowledged, that the Foekens et al. cohort includes almost double as many patients as our cohort. However, by using the paired design we increased the prognostic power for our analysis as discussed above.

In conclusion, using data of four independent cohorts analyzed by different analysis platforms we collected further indications supporting the prognostic potential of hsa-miR-548c-5p, hsa-miR-7-5p, hsa-miR-210-3p and hsa-miR-128-3p. In addition, a potentially time-dependent role was determined for hsa-miR-516a-3p. We exclusively selected systemically untreated, LN negative and ER positive breast cancer patients for a treatment independent analysis. The stringent selection limited the number of patients to be analyzed and may reduce the prognostic power of the study, however, only this particular patient group is suitable to identify miRs which can better classify low-risk breast cancer patients and thus have the potential as clinical marker to reduce overtreatment in the future. Nevertheless, further studies in large, homogeneous cohorts with standardized sample treatment and long follow up using accurate and selective platforms are required to eventually confirm the prognostic potential of these candidates for clinical use.

MATERIALS AND METHODS

Tumor biopsies

Frozen tumor biopsies were collected from 110 lymph node negative and ER positive (measured by gene expression) patients who were diagnosed with breast cancer between 1980 and 1999 on the island of Funen, Denmark. All patients underwent surgery to remove the primary tumor, but none of the patients received systemic neoadjuvant or adjuvant therapy. Pathological examination of snap-frozen tumor samples was performed at the Department of Pathology at the Odense University Hospital, Denmark which confirmed a tumor cell content of more than 50% in all cases. Fifty-five patients developed
metastasis within 10 years after diagnosis, while 55 patients
did not experience metastasis (followed for at least 8 years;
mean follow up 19.6 years). Patient biopsies were matched
according to tumor type, year of surgery, tumor diameter
(range: 6–50 mm), age (range: 33–88 years), receptor status
(ER, PR, n/a) and histological grade (grade 1–3 or n/a)
(Table 1). All clinic-pathological information was extracted
from the Danish Breast Cancer Cooperative Group (DBCG)
database, the Funen pathology database, or the nationwide
pathology database. The study was approved by the Danish
National Committee on Health Research (S-VF-20020142).
The study was retrospective and no informed consent
was obtained from the patients involved in the study as
approved by the Ethical Committee.

RNA extraction

Total RNA was isolated from the freshly frozen
tumor biopsies with Trizol (Invitrogen) and further purified
with the RNeasy micro kit (Qiagen), including DNase
treatment. NanoDrop Spectrophotometer (NanoDrop
Technologies) was used for RNA quantification. The
quality of extracted RNA was assessed with Bioanalyzer
2100 (Agilent Technologies) using the RNA 6000 Nano
Kit (Agilent Technologies).

Microarray quantification and analysis

The miRCURY LNA microRNA array ready-to-
spot probe-set was purchased from Exiqon. The set
contains capture-probes for 1212 human miRs based
on the miRBase version 16 (http://www.mirbase.
org/) [19]. Briefly, the probes were spotted in triplets
on CodeLink HD (SurModics) activated glass slides and
post-processed according to the CodeLink
protocol. The miRCURY Power labeling kit (Exiqon),
miRCURY LNA Array hybridization buffer kit (Exiqon)
and miRCURY LNA Array Washing buffer kit (Exiqon)
were used for sample labelling with Hy3, hybridization
and washing, respectively. Equal amounts of RNA from
all included samples were pooled and used as common
reference labeled with Hy5. Hybridization, washing, and
scanning (Agilent G2565CA Microarray scanner) were
performed according to the recommendations provided
by Exiqon.
Scanned images were imported into GenePixPro6.0
software (Molecular Devices) for quality control and
raw data extraction. The R-package limma was used for
LOESS and quantile normalization of raw signal
intensities, and the ComBat function embedded in the
sva R-package was used for adjustment of the normalized
intensities and to eliminate potential batch effects [46, 47].
Gene expression was initially defined as the ratio between
Hy3 and Hy5 signal intensities. For the purpose of
cross dataset miR expression comparability we further
standardized these calculated ratios and used these
values for differential expression and survival analysis.
Microarray data have been deposited in NCBI’s Gene
Expression Omnibus [48] and are accessible through GEO
Series accession number GSE103161 (https://www.ncbi.
nlm.nih.gov/geo/query/acc.cgi?acc=GSE103161).

Validation data acquisition

Publicly available miR expression data published
by D’Aiuto et al. were collected from NCBI’s Gene
Expression Omnibus database (acc=GSE59829) [48].
Expression data published by Dvinge et al. and Curtis
et al. (Metabric) were retrieved from the European
Phenome-Genome Archive (https://www.ebi.ac.uk/ega/
home) after access was granted by the Metabric consortium
[15, 16, 49]. For consistent miR nomenclature the
R-package miRNAmeConverter was applied and MIMAT
numbers were used as identifier in subsequent analysis [50].

Statistical analysis

Student’s t-tests were performed to analyze
differential expression. For each of the seven miRs
we divided the samples into groups with high and low
expression using the median expression as cut off. The
grouping was used for subsequent analyses using the COX
univariable proportional hazard model [22].
Overall survival (OS) was defined from diagnostic
date to death or loss of follow-up. Disease free survival
(DFS) was defined from diagnostic date to metastasis
recurrence or loss of follow-up. The Kaplan–Meier
method was applied to describe the OS and DFS.
Significant discrepancies of OS or DFS were assessed
by univariable COX proportional hazard analysis [22, 51].
The proportional hazards assumption and time-dependent
effects were examined by plotting the scaled Schoenfeld
residuals to test for significant deviation from a zero
slope, respectively [21]. Landmark analyses were applied
to assess the hazard ratios during particular periods. The
landmarks were defined as the time point upon which the
Schoenfeld assessed Hazard Ratio (HR) shifts from >1 to
<1, thus being (0–3.35 years and >3.35 years for DFS)
and (0–4.5 years and >4.5 years for OS). This analysis
therefore included patients who were event free at the
start of each period, and being censored at the end of each
period. All survival analyses were conducted using the
survival R-package [22, 51]. All p-values below 0.05 were
considered statistically significant.

Abbreviations

OS: Overall survival; DFS: Disease free survival;
ER: estrogen receptor; LN: lymph node; PR: progesterone
receptor: ERBB2: erb-b2 receptor tyrosine kinase 2; miR:
microRNA; Metabric: Molecular Taxonomy of Breast
Cancer International Consortium; DBCG: Danish Breast
Cancer Cooperative Group; OUH: Odense University Hospital; Std. expression: Standardized expression; HR: Hazard ratio; CI: Confidence interval; STDEV: Standard deviation.

Author contributions

KPS, IB, MT and TAK conceived the study and designed the experiments; KPS and MJL performed the experiments; MBU performed the data analysis; IB, MJL, QT and LA contributed to the data analysis; TAK, MT, QT, MBU, IB interpreted the results; MBA and SC contributed materials, patient information and supported the interpretation of the results. IB wrote the manuscript. All authors revised the manuscript and approved the submitted version.

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CONFLICTS OF INTEREST

The authors declare that no competing interests exist.

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