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Prospective validation of a blood-based 9-miRNA profile for early detection of breast cancer in a cohort of women examined by clinical mammography

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ABSTRACT

Mammography is the predominant screening method for early detection of breast cancer, but has limitations and could be rendered more accurate by combination with a blood-based biomarker profile. Circulating microRNAs (miRNAs) are increasingly recognized as strong biomarkers, and we previously developed a 9-miRNA profile using serum and LNA-based qPCR that effectively stratified patients with early stage breast cancer vs. healthy women. To further develop the test into routine clinical practice, we collected serum of women examined by clinical mammography (N = 197) according to standard operational procedures (SOPs) of the Danish Cancer Biobank. The performance of the circulating 9-miRNA profile was analyzed in 116 of these women, including 36 with breast cancer (aged 50−74), following a standardized protocol that mimicked a routine clinical set-up. We confirmed that the profile is significantly different between women with breast cancer and controls (p-value <0.0001), with an AUC of 0.61. Significantly, one woman whose 9-miRNA profile predicted a 73% probability of having breast cancer indeed developed the disease within one year despite being categorized as clinically healthy at the time of blood sample collection and mammography. We propose that this miRNA profile combined with mammography will increase the overall accuracy of early detection of breast cancer.

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Abbreviations: ER, estrogen receptor; HER2, human epidermal growth factor 2; miRNA, microRNA; SOP, standard operational procedures; LNA, locked nucleic acid; AUC, area under the curve; ROC, receiver operating characteristic.

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1. Introduction

Mammography is currently the standard screening method for breast cancer in many countries, fulfilling the WHO criteria (Chestnov et al., 2014). However, the method has limitations due to relatively high false-positive rates, and limitations in detecting smaller tumors, including those in dense breast tissue (Nielsen et al., 2016a,b). In 2014, WHO published an updated position on mammography screening (Chestnov et al., 2014) to provide independent guidance on the balance between benefits and harms in women of different age groups. In well-resourced settings, such as many Western countries, biennial screening is suggested for women aged 50–69 years (Mittmann et al., 2015), which is the current screening interval, and age-range, in Denmark.

Mammograms are increasingly becoming digitalized (computerized) and being developed further as a new tomographic technique, termed digital breast tomosynthesis (DBT) (Gilbert et al., 2016). Despite these technological developments, however, there are still limitations resulting in false-positive and -negative results, and implementation of these advanced digitalized techniques is costly and consequently difficult in many countries. Nuclear breast imaging in which radiotracers are used for functional screening rather than X-ray-based anatomic screening (Berg, 2016) has been tested in women with a high-risk of developing breast cancer, and has potential for population-based screening, although likely not in the near future.

Thus, there is great interest in identifying circulating biomarkers to screen for early stage cancer using blood of seemingly healthy individuals. These circulating biomarkers include nucleic acid fragments shed into the blood stream from cancerous cells, either apoptotic or necrotic, or as an active secretory process (Schwarzenbach et al., 2014). The notion of a “liquid biopsy” has several advantages since circulating nucleotides (miRNA/DNA) are highly stable in the blood-stream (Kosaka et al., 2010; Mitchell et al., 2008), likely represent the entire tumor vs. an isolated tumor needle biopsy, permit automation of most of the analysis, and support compliance due to minimal discomfort. The presence of miRNA in the blood of cancer patients is believed to have a half-life ranging from 15 min to several hours, similar to cell-free DNA (Schwarzenbach et al., 2014; Minchin et al., 2001; Botezatu et al., 2000), providing continuous markers of the cancer applicable to screening as well as monitoring potential recurrence, and perhaps also serving as early markers of the effect of certain cancer treatments.

The first report of miRNAs in the blood stream in 2008 (Chim et al., 2008; Lawrie et al., 2008) initiated an intense search for these markers for various diseases, but enthusiasm for using miRNAs has been challenged in recent years (Witwer, 2015; Schwarzenbach et al., 2014). Issues raised include the risk that miRNAs may represent the body’s “danger” response to a cancer more than the tumor itself. There are also limitations with reproducibility of miRNA profiles attributed to variability of pre-analytical handling, population diversity and varying technologies used. Despite these concerns, development in this field continues, since blood-based biomarker assays have great clinical potential and miRNAs are excellent candidates.

We previously reported the identification and retrospective validation of a circulating 9-miRNA profile for detection of early stage breast cancer using miRNAs isolated from serum samples obtained in the late 90’s from 48 women with early breast cancer and 24 healthy controls. Serum samples from an additional cohort of 111 women (60 with early stage breast cancer and 51 controls) were used for validation and confirmed the ability of the 9-miRNA profile to distinguish between women with breast cancer and healthy individuals (Kodahl et al., 2014).

To further develop this assay for clinical use, we evaluated the blood-based 9-miRNA profile of a prospective cohort of women undergoing clinical mammography (N = 197). This cohort was comprised of 18% invasive cancer patients (N = 36), and 82% clinically healthy controls (N = 161), with an age-range of 50–74 years. Every second control was chronologically selected to obtain a 2:1 ratio (controls to cases), resulting in miRNA profiling of 116 women. Our finding supports the utility of the 9-miRNA profile for early detection of breast cancer, which would allow initiation of treatment at an earlier time point.

2. Materials and methods

2.1. Breast cancer patients and healthy controls

Women undergoing clinical mammography due to recall from screening mammography were asked to participate in the study, and upon signing the informed consent, blood was drawn from 197 women (aged 50–74 years) from October 2013 to July 2015. For miRNA profiling, all invasive breast cancer cases were included (N = 36), and every second chronological database entry of a woman with no mammography-detected breast cancer was selected for the control group (N = 80), resulting in a 2:1 ratio of healthy controls vs. breast cancer cases. Breast cancer diagnosis was confirmed by biopsy and surgical specimen. Follow-up of healthy controls consisting of review of medical files and/or new mammograms was performed in April 2016; follow-up period: 0.7–2.4 years. Blood samples were collected prior to clinical mammography and serum was isolated according to SOPs of the Danish Cancer Biobank (DCB Herlev Hospital, 2016). Briefly, serum was prepared within 1 h of blood collection by coagulation between 30 and 120 min, followed by centrifugation at 4 °C, 2000 g for 10 min, and immediately stored after fractionation at −80 °C. Clinical characteristics of those included in the statistical analysis are listed in Table 1. The study is approved by the Regional Ethical Committee (ID: S-20100132), and all participants signed informed consent. The REMARK guidelines were followed where possible (McShane et al., 2005).

2.2. Isolation of RNA from serum

Total RNA was extracted from serum using the miRCURY™ RNA isolation kit (Exiqon, Denmark), including addition of 1 μg carrier-RNA per 60 μL lysis and finally eluted in 50 μL...
2.3. miRNA real-time PCR

Reverse transcription was conducted with 2 μL RNA in 10 μL reactions using the miRCURY LNA™ Universal RT microRNA PCR, Polyadenylation and cDNA synthesis kit (Exiqon) in technical triplicates. cDNA was diluted ×50 and assayed in 10 μL PCR reactions according to the protocol for miRCURY LNA™ Universal RT microRNA PCR; each microRNA was assayed once by qPCR on the microRNA Ready-to-Use PCR, custom Pick and Mix Panel using ExiLENT SYBR® Green master mix (Exiqon). Negative controls excluding template from the reverse transcription reaction was performed and profiled in the same manner as the samples. Amplification was performed in a LightCycler® 480 Real-Time PCR System (Roche, Germany) in 384 well plates, and the amplification curves were analyzed using the Roche LC software both for determination of Cq (by the 2nd derivative method) and for melting curve analysis.

2.4. Sample quality

Raw data and all assays were inspected for distinct melting curves and the Tm was verified to be within known specifications for the assay. Only assays detected with 3 Cqs less than the negative control, and with Cq < 37, were included in the data analysis. DNA spike-ins’ were used to assess the quality of the RNA extraction, RT and qPCR steps. These assays were not used for normalization. Whether hemolysis had occurred in the blood samples was evaluated by determining the ratio of miRNA-451 (expressed in red blood cells) and miRNA-23a (stable in blood, and not affected by hemolysis) (Blondal et al., 2013) and samples exhibiting a ratio of > 7 were excluded.

2.5. Normalization and data analysis

In our previous publication, miRNA-10b-5p was identified to be the most stable miRNA, based on 65 miRNA assays, and therefore used as reference miRNA. The risk score was calculated using the normalized ($\Delta C_{q,\text{target}} = \log_2 (C_p \text{ target miRNA}) - \log_2 (C_p \text{ miRNA-10b-5p})$) miRNA values, centered and standardized to a standard deviation of 1, of the 9 miRNAs (miR-15a, miR-18a, miR-107, miR-133a, miR-139-5p, miR-143, miR-145, miR-365, miR-425) and the following equation: $\text{score} = 0.934 + (-0.152 \cdot \Delta C_p \text{ [miR-143-3p]} + (-0.435 \cdot \Delta C_p \text{ [miR-139-5p]} + (0.341 \cdot \Delta C_p \text{ [miR-107]} + (-1.124 \cdot \Delta C_p \text{ [miR-145-5p]} + (0.702 \cdot \Delta C_p \text{ [miR-15a-5p]} + (0.31 \cdot \Delta C_p \text{ [miR-425-5p]} + (0.18 \cdot \Delta C_p \text{ [miR-18a-5p]} + (-0.275 \cdot \Delta C_p \text{ [miR-133a-3p]} + (-0.134 \cdot \Delta C_p \text{ [miR-365a-3p]}). This score can be converted into a predicted probability of having breast cancer by the equation $L = 1/(1 + \exp(-\text{score}))$. Comparison between groups was performed by means of t-tests; discrimination performance was assessed using ROC curves and the corresponding area under the curve (AUC).

3. Results

3.1. Patient characteristics

A consecutive cohort of women (N = 197) who had undergone clinical mammography due to recall from screening mammography was evaluated, 36 of which had invasive breast cancer. The controls were then selected to obtain a 2:1 ratio of controls to cases, choosing every second woman included chronologically in the study, leading to a total of 116 samples for miRNA profiling. The clinical characteristics of these participants are summarized in Table 1. Since our previous study identified no difference in miRNA expression according to lymph node involvement, age, menopausal status or tumor size between breast cancer samples, these parameters were not used to further stratify the patients. The qPCR data generated was analyzed as in our previous study (Kodahl et al., 2014), including stringent quality control measures. Two serum samples, both from individuals in whom no breast cancer was detected by clinical mammography, were excluded due to the risk of hemolysis to eliminate any potential influence on our results.

3.2. Clinical utility of the 9-miRNA profile

The 9-miRNA profile was significantly different between patients with ER+ breast cancer and healthy controls (p < 0.0001, (95% CI [0.41; 0.86])) with an AUC of 0.58 (Figure 1A) following a completely standardized flow of analysis, confirming our previously published results. We also investigated the performance of the profile for the complete dataset, including the ER – breast cancer samples (Figure 1B).
This profile also reached significance (p < 0.0001, 95% CI [0.35; 0.77]), and there was a slight increase in the AUC (0.61) for early detection of combined ER+ and ER− breast cancer.

The qPCR data for the 9-miRNAs can be transformed into a risk score that can be converted mathematically to a predicted probability of breast cancer (Figure 2). This predicted probability functions as a continuous value, and its performance was evaluated by reviewing whether any of the women in whom no breast cancer was detected by clinical mammography subsequently developed breast cancer within a follow-up period of 0.7−2.4 years. One woman was diagnosed with bilateral breast cancer 348 days after blood sampling, and did not have a biopsy taken at clinical mammography. Her 9-miRNA profile score was 0.978, resulting in a predicted probability of having breast cancer of 0.73 (i.e. 73%).

To assess the sensitivity and specificity of the 9-miRNA profile, a cut-off of 0.56 (risk score value) was chosen, yielding 0.70 and 0.47, or 0.73 and 0.44, respectively, for the ER+ group only or the combined ER+ and ER− group.

4. Discussion

In this study, we verified our circulating 9-miRNA profile (Kodahl et al., 2014) in a prospectively collected cohort of post-menopausal women undergoing clinical mammography due to recall after screening mammography. A ratio of 2:1 of clinically healthy controls vs. breast cancer patients, respectively, was chosen to minimize the potential risk of bias, as some of the healthy controls may have clinically undetectable cancer or inflammation that could potentially affect the results. The set-up of this study mimicked a diagnostic test in a standard clinical setting, following clear SOPs and core facility techniques.

Although mammography is a widely used and approved method for detecting breast cancer, it is not optimal since the test can be affected by breast density, is operator-dependent and is quite uncomfortable. Twenty seven percent of the women included in our cohort had a biopsy taken at time of the clinical mammography (N = 53/197), and 68% (N = 36) were found to have cancer. The women selected for biopsy were not only based on X-ray mammography, but also clinical exams and ultrasound. Screening mammography has a high percentage of recall, and most turn out not to have cancer. In a recent American meta-analysis (2016), a 42% cumulative rate of false-positives over 10 years were found upon biennial screening (Nelson et al., 2016a,b). Furthermore, the invasive lobular carcinomas (ILCs) have long been recognized as difficult to image by X-ray due to the growth pattern of diffuse infiltration of single rows of malignant cells, which does not destroy the underlying anatomic structures (Johnson et al., 2015). A normal distribution (Lakhani et al., 2016) of invasive ductal carcinomas (IDCs) (80%) and ILCs (11%) was observed in this study.

Cell-free (cf) miRNAs have all the characteristics of an optimal blood-based cancer biomarker, such as stability in both the blood stream and during handling and storage, and likely reflect the tumor in its entirety compared to a single biopsy. Cell-free miRNAs have been determined to be RNase-resistant in plasma as they were found to be bound with complexes of the protein AGO2, but a minority were also found to

![Figure 1](image1.png)  
**Figure 1** — ROC curve analysis using the 9-miRNA profile for discriminating breast cancer cases from controls (N = 78), i.e. without a suspicious finding on the mammogram. A) Graph only including the ER+ breast (N = 31) cancer cases, with an AUC = 0.58 (p < 0.0001, 95% CI [0.41; 0.86]). B) Graph of the ER+ and ER− breast cancer samples combined (N = 36), having an AUC = 0.61 (p < 0.0001, 95% CI [0.35; 0.77]).

![Figure 2](image2.png)  
**Figure 2** — Visualization of the relation of the risk score to the predicted probability of having breast cancer. The score was calculated from the normalized qPCR expression data of the 9 miRNAs using the formula provided in Material and Methods.
be associated with vesicles or exosomes (Mitchell et al., 2008; Mathivanan et al., 2010).

The published miRNA blood-based profiles for detection of early breast cancer have minimal overlap in which miRNAs are included (Ng et al., 2013; Zhu et al., 2009; Cuk et al., 2013; Heneghan et al., 2010; Zhang et al., 2015; Freres et al., 2016; Haggrass et al., 2015; Mishra et al., 2015). This lack of consistency could be due to redundancy in miRNA function, and several miRNAs could be deregulated in the same manner under certain conditions, replacing one miRNA with another. Another explanation for the lack of overlap could be the technical approach, from the pre-analytical techniques of blood sampling and isolation of serum/plasma, to the use of assays (qPCR, array or sequencing) and reagents.

All women in this validation study were above the age of 50 and thus most likely post-menopausal, representing the common mammography screening population. Our profile was previously developed, including thorough investigation of the normalization procedure, and we could significantly identify invasive breast cancer cases among healthy controls (Kodahl et al., 2014). This was underscored by one woman with a 73% predicted probability of breast cancer who had no evidence of breast cancer at the time of clinical mammography and blood sample, but in whom a palpable lump and a breast cancer diagnosis was confirmed nearly a year later. Based on our blood test, this individual could potentially have undergone further medical exams and had treatment initiated much earlier. As our population was restricted in available ER—breast cancer samples, we will need to further investigate this in a larger ER—breast cancer population to confirm the all-round performance of this 9-miRNA profile. Investigations of non-malignant breast lesions to identify the potential to discriminate between these and invasive breast cancer will also be required. Furthermore, it would be very interesting to examine the profile in younger women, as their denser breast tissue poses a challenge for mammography.

5. Conclusion

Overall, while screening mammography has generally been shown to reduce breast cancer mortality, a recent study indicates that the effect may only be borderline statistically significant, particularly for younger women (Nelson et al., 2016a,b). MicroRNAs are very potent blood-based biomarkers, but their use in the clinical setting is still limited due to lack of reproducibility, limited sample sizes investigated and the lack of independent validation cohorts. Ideally, a blood-based biomarker profile combined with mammogram findings could increase both sensitivity and specificity and render an earlier, more precise, diagnosis of breast cancer. This potential of the 9-miRNA profile was underscored by the subsequent identification of an interval breast cancer in one woman who, in our initial analysis, had a 73% risk of breast cancer based on our 9-miRNA profile blood test, but no detectable disease at the time of mammography and blood sampling. As the data matures, we will investigate whether more interval cancers are diagnosed, and further assess the miRNA profile to see whether the 9-miRNA profile could have indicated the presence of a mammographically-undetectable cancer.

Authors’ contribution

MBL, ARK and HJD participated in the study design. MBL coordinated the project, performed data interpretation and wrote the first draft of the manuscript. HB performed the statistical analysis. ARK, HB and HJD assisted in writing the manuscript, and all authors have read and approved the final manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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References

Paper on Mammography Screening. [http://apps.who.int/iris/bitstream/10665/137339/1/9789241507936_eng.pdf?ua=1&ua=1]


DCB Herlev Hospital, 2016. The Danish Cancer Biobank. [http://www.danskcancerbiobank.dk/]


