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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

Maria B. Lyng[a,*], *Annette R. Kodahl*[b], *Harald Binder*[c], *Henrik J. Ditzel*[a,b,**]

[a]*Institute of Molecular Medicine, University of Southern Denmark, Odense, Denmark*
[b]*Department of Oncology, Odense University Hospital, Odense, Denmark*
[c]*Institute of Medical Biostatistics, Epidemiology and Informatics (IMBEI), University Medical Center, Johannes Gutenberg University, Mainz, Germany*

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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.

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.

* *Corresponding author*. Institute of Molecular Medicine, University of Southern Denmark, J.B. Winsløws Vej 25, 3, 5000 Odense C, Denmark.

** *Corresponding author*. Institute of Molecular Medicine, University of Southern Denmark, J.B. Winsløws Vej 25, 3, 5000 Odense C, Denmark.

E-mail addresses: maria.lyng@rsyd.dk (M.B. Lyng), hditzel@health.sdu.dk (H.J. Ditzel).

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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 (Nelson 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 harm 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

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