Serum miR-181a and miR-25 levels in patients with breast cancer or a benign breast disease

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Abstract

Breast tumours comprise a wide range of disorders requiring different and often personalised treatment plans. The microRNA levels indicating the regulation of gene expression involved in oncogenesis can serve as diagnostic and prognostic biomarkers of benign and malignant breast diseases. Circulating miR-181a and miR-25 were quantified here using droplet digital PCR (ddPCR) in 77 serum samples from patients with invasive breast carcinoma (IBC) (50 samples) or benign breast diseases (16 samples) and ‘potentially healthy’ controls (11 samples). MiR-181a expression was higher in patients with fibroadenoma or fibrocystic disease/adenosis (low risk of malignant transformation) as compared to potentially healthy controls. In IBC patients, miR-181a expression was higher in luminal B-like (HER2−), HER2+ (non-luminal) and triple-negative breast cancer (TNBC) groups, while miR-25 expression was higher in luminal B-like (HER2+) and TNBC groups compared to potentially healthy controls. Compared to the luminal A-like group, miR-181a expression was higher in luminal B-like (HER2−) and HER2+ (non-luminal) groups, whereas miR-25 expression was elevated in luminal B-like (HER2+) and TNBC groups. MiR-25 expression was higher in the luminal B-like (HER2−) group compared to the TNBC group. Thus, miR-181a and miR-25 may be markers of precancerous changes in women with benign breast diseases. In IBC patients, levels of miR-181a and miR-25 can reflect either favourable or adverse processes in a tumour owing to their multiple effects. They can be potentially used as biomarkers in a large diagnostic panel.

Introduction

Breast cancer (BC) is the most common cancer among women and the leading cause of cancer death1,2. Invasive breast carcinoma (IBC) includes a wide range of malignant epithelial tumour subtypes that vary in morphology, clinical presentation and prognosis and require different, often personalised treatment plans1,2.

IBC aetiology and pathogenesis may be related to dysregulation of microRNAs (miRNA, miR)3, which repress their target genes at the post-transcriptional level. Many of these genes are involved in cell proliferation, differentiation, migration, and apoptosis3,4. It is expected that miRNA expression profiling will allow them to be employed as biomarkers for diagnosis, theranostics and prognosis5.

MiR-181a and miR-25 are known as either oncogenic miRNAs or tumour suppressors in different types of cancer5–7, in different types of BC3,4 and even within the same BC subtype8–10. In breast tissues, miR-181a as a tumour suppressor targets genes encoding matrix metalloproteinase MMP-1411, pleckstrin homology-like domain, family A, member 1 (PHLDA1), BC resistance protein BCRP4, Bcl-212 and autophagy-associated proteins13. As a proto-oncogene, miR-181a is associated with aberrant activation of the TGF-β signalling pathway4 and targets mRNA of genes ataxia telangiectasia mutated (ATM), BAX4 and NDRG23. MiR-181 enhances metastatic potential of BC cells, thereby promoting epithelial–
mesenchymal transition and formation of an invasive phenotype\textsuperscript{14}, whereas high levels of miR-181a correlate with poor survival of patients with BC\textsuperscript{15}.

MiR-181a is one of the most common exosomal human plasma miRNAs\textsuperscript{16}, and changes in its serum level may serve as a biomarker\textsuperscript{4}. Nonetheless, the data available on serum miR-181a levels depending on disease status are inconsistent\textsuperscript{17,18}.

MiR-25 is mostly known as an oncogenic miRNA\textsuperscript{19}. MiR-25 expression is elevated in BC samples compared to non-malignant breast tissues in aggressive BC types such as triple-negative BC (TNBC)\textsuperscript{20} and HER2\textsuperscript{+} BC\textsuperscript{5}. MiR-25 promotes tumour proliferation by targeting tumour suppressor \textit{BTG2}\textsuperscript{21}’s mRNA in TNBC and participates in autophagy processes by interacting with autophagy regulator \textit{ULK1}\textsuperscript{22}’s mRNA and in TNF-dependent cell death by interacting with \textit{NOX4} mRNA\textsuperscript{23}. Nevertheless, there is also evidence of improved survival in BC with elevated miR-25 levels\textsuperscript{24} as well as tumour growth inhibition via the involvement of miR-25 in the regulation of the Wnt signalling pathway\textsuperscript{25}.

Overexpression of miR-25 has been shown in the serum of Chinese BC patients\textsuperscript{26}, and it can be reasonably assumed that miR-25-3p is a biomarker of BC.

Unfortunately, little attention has been paid to the treatment of benign breast diseases\textsuperscript{27}, although these conditions may increase the risk of BC\textsuperscript{28}. For example, a complex breast cyst and complex solid and cystic breast mass can be malignant in 23–31\% of cases\textsuperscript{29}. Adenosis is often associated with fibrocystic alterations, whereas sclerosing and apocrine adenosis correlate with a 1.5–2.0-fold increase in the risk of BC\textsuperscript{2}. Fibroadenoma is generally associated with a minimal increase in the risk of malignancy\textsuperscript{2}; however, the risk is high in women with a family history of BC and/or BRCA-1/2 mutations\textsuperscript{30}.

An analysis of circulating miR-181a miR-25 levels in different types of breast disease can provide a clearer understanding of their potential as biomarkers. With this aim, we investigated concentrations of miR-181a and miR-25 in the serum of patients with IBC of different molecular subtypes, with or without lymphogenous metastasis, as well as in patients with a benign breast disease and in potentially healthy donors.

**Materials And Methods**

**Patients and sample collection**

The study population consisted of 77 serum samples collected from patients at Municipal Hospital No. 1, Novosibirsk (Russia). Eleven people (Novosibirsk Municipal Blood Transfusion Station) were classified as potentially healthy, 50 patients got a diagnosis of IBC (among them, 48 patients with stage GII and two patients with stage GI) and 16 patients had a benign breast disease. Metastases in regional lymph nodes
were present in 20 IBC patients (average age 52 [range 23–72] years) and absent in 30 patients (average age 58 [range 35–79] years).

The whole study was conducted in accordance with the World Medical Association Declaration of Helsinki 1964 as amended in 2013 at the 64th WMAJ General Assembly (Fortaleza, Brazil, October 2013). All patients gave their voluntary informed consent to participate in the study. The study protocol was approved by the Ethics Committee at the Institute of Molecular Biology and Biophysics, a subdivision of the Federal Research Centre of Fundamental and Translational Medicine (Protocol No. 2016-3).

The patients underwent surgical treatment and finally got a diagnosis on the basis of pathomorphological findings. Neoadjuvant therapy was not performed.

In recent years, new molecular markers for differences in pathogenesis, treatment response and prognosis were added to the IBC classification. Nevertheless, due to the lack of time and resources, molecular classification of BC in the vast majority of healthcare systems is still largely based on immunohistochemical evaluation of such biomarkers as oestrogen receptor (ER), progesterone receptor (PR) and epidermal growth factor receptor 2 (HER2) as well as on assays of the Ki-67 proliferation marker. We chose this classification and identified molecular subtypes of each tumour among the samples under study (Table 1).

<table>
<thead>
<tr>
<th>Subtype</th>
<th>ER</th>
<th>PR</th>
<th>HER2</th>
<th>Ki-67 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal A-like</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
<td>&lt; 20%</td>
</tr>
<tr>
<td>Luminal B-like HER2⁻</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
<td>≥ 20%</td>
</tr>
<tr>
<td>Luminal B-like HER2⁺</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>Any</td>
</tr>
<tr>
<td>HER2⁺ (non-luminal)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Any</td>
</tr>
<tr>
<td>TNBC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Any</td>
</tr>
</tbody>
</table>

Luminal A-like BC was diagnosed in 15 patients with IBC [average age 58 (35–79) years], luminal B-like HER2 negative (luminal B-like [HER2⁻]) in 12 patients [average age 53 (23–69) years], luminal B-like HER2⁺ (luminal B-like [HER2⁺]) in 2 patients [average age 57 (47–67) years], HER2⁺ (non-luminal) type in 5 patients, [average age 55 (40–69) years], and TNBC was diagnosed in 14 patients [average age 56 (35–72) years].

Patients with a benign breast disease were subdivided into two groups. The first group of a low risk of malignant transformation (a non-proliferative type of fibrocystic disease, fibroadenosis or fibroadenoma) included 14 patients [average age 50 (18–83) years]. The second group included two patients: a 40-year-old woman with a proliferative type of fibroadenosis and a 41-year-old woman with sclerosing adenosis.
Rna Isolation And Cdna Synthesis

MiRNA was isolated using the NucleoSpin miRNA Plasma Kit (Macherey-Nagel, Germany) as per the manufacturer's protocol. Reverse transcription was performed with miRNA-specific stem-loop adapters and reverse transcriptase M-MuLV–RH (Biolabmix, Russia), as per the manufacturer's protocol. The mixture was incubated at 18 °C for 30 min, then at 42 °C for 30 min and finally at 85 °C for 5 min. Sequences of the stem-loop adapters were as follows: miR-181a: 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACTCACCG-3′; miR-25: 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAGACCG-3′; U6: 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGACTGGATACGACGGCCATGC-3′.

Droplet Digital Pcr (Ddpcr)

MiRNA expression was evaluated by means of ddPCR probes and a QX200 AutoDG Droplet Digital PCR System (Bio-Rad Laboratories, USA). To generate droplets in a nal volume of 20 µl, 2x supermix for ddPCR (Bio-Rad), 7 µl cDNA and a primer mix consisting of 5 µM probe and 20 µM forward and reverse primers were used. The primer sequences were as follows: miR-181a: forward 5′-GCCGCAACATTCAACGCTGT-3′, probe 5′-(FAM)-TTCGCACTGGATACGACACTCACCG-(BHQ1)-3′; miR-25: forward 5′-GCCGCCATTGCACTTGTCT-3′, probe 5′-(FAM)-TTCGCACTGGATACGACTCAGACCG-(BHQ1)-3′; U6: forward 5′-GCCGCATACAGAGAAGATTA-3′, probe 5′-(FAM)-TTCGCACTGGATACGACGGCCATGC-(BHQ1)-3′; and common reverse primer 5′-AGTGCAGGGTCCGAGGTATTCGACTGGATACGACGGCCATGC-3′.

Droplets were obtained using a QX200 automatic droplet generator (Bio-Rad). The reaction was conducted under the following conditions: heating at 95°C for 10 min, then 39 cycles of denaturation at 95°C for 30 s and annealing/extension at 55°C for 10 min, then 98 °C for 10 min. After that, a QX200 droplet reader was used, and the results were analysed in the Quantasoft™ software (Bio-Rad). A no-matrix control was included in each assay. Small nuclear RNA U6 served as an internal standard for the miRNAs being quantified.

Statistical analysis

This procedure was performed using the STATISTICA software. The data distribution pattern was determined by the Lilliefors-corrected Kolmogorov–Smirnoff test. The Kruskal–Wallis test was performed to compare independent groups, followed by an intergroup comparison using the Mann–Whitney U test.

Results And Discussion

The quantitation of miR-181a and miR-25 levels in the serum of patients with IBC of different molecular subtypes showed that the samples of aggressive IBC subtypes tend to have higher levels of miR-181a and miR-25. The obtained differences were significant according to the Kruskal–Wallis test at p = 0.0127 for miR-181a (Fig. 1) and p = 0.0077 for miR-25 (Fig. 2).
MiR-181a and miR-25 in IBC samples

Pairwise comparisons of expression levels among the groups by the Mann–Whitney $U$ test indicated that miR-181a expression was higher in luminal B-like (HER2−) ($p = 0.006707$), HER2+ (non-luminal) ($p = 0.017358$) and TNBC groups ($p = 0.017247$) compared to potentially healthy controls (Fig. 1), whereas miR-25 expression was found to be elevated in luminal B-like (HER2−) ($p = 0.004346$) and TNBC groups ($p = 0.030586$) (Fig. 2).

Compared to the luminal A-like group, miR-181a expression was higher in luminal B-like (HER2−) ($p = 0.021335$) and HER2+ (non-luminal) groups ($p = 0.023241$) (Fig. 1), whereas miR-25 expression proved to be elevated in luminal B-like (HER2−) ($p = 0.001175$) and TNBC ($p = 0.038167$) groups (Fig. 2). In addition, the miR-25 level was higher in the luminal B-like (HER2−) group than in the TNBC group ($p = 0.043372$) (Fig. 2).

Despite some evidence of a relation between metastases and miR-181a levels\textsuperscript{14,35}, we failed to reveal any significant differences either in the miR-181a level or in the miR-25 level between the groups of patients with and without metastases to lymph nodes. Furthermore, we did not find any correlations between the levels of studied miRNAs and age; this finding may be important in terms of immunosenescence\textsuperscript{36}.

MiR-181a and miR-25 in luminal A-like samples

Luminal A-like BC is the most common molecular subtype of BC with a relatively good prognosis\textsuperscript{37}. In our work, levels of both miR-181a and miR-25 in the luminal A-like BC group did not differ from those in the group of potentially healthy subjects and were the lowest in the non-control groups. Thus, miR-181a and miR-25 do not seem to affect the pathogenesis of luminal A-like IBC as either proto-oncogenes or tumour suppressors.

MiR-181a and miR-25 in luminal B-like samples

The highest level and widest range of miR-181a and miR-25 expression values were observed in the luminal B-like (HER2−) IBC group. Compared to luminal A-like IBC, these tumours have a more aggressive phenotype, a higher risk of recurrence and a worse prognosis\textsuperscript{38}. The luminal B-like BC phenotype features the greatest extent of tumour genome methylation among all BC subtypes\textsuperscript{39}. On the one hand, alterations in the epigenome cause miRNA dysregulation in cancer; on the other hand, miRNAs themselves indirectly control these DNA and histone modifications. Increased miR-181a and miR-25 expression levels may reflect methylation processes in luminal B-like IBC cells\textsuperscript{40}.

MiR-181a and miR-25 in HER2+ samples

In agreement with literature data\textsuperscript{41}, miR-181a expression was found to be elevated in HER2+ (non-luminal) IBC samples. We can also noticed a trend towards higher miR-181a levels in luminal B-like (HER2+) samples; however, we cannot draw conclusions from the analysis of two samples.
MiR-181a has been reported to suppress the ATM gene in BC cells, thereby impairing the DNA damage response\(^8,^{42}\). ATM dysfunction contributes to HER2-dependent carcinogenicity \textit{in vitro} and \textit{in vivo}\(^43\). On the other hand, ATM may have tumourigenic potential in HER2\(^+\) BC as a modulator of the HER2 protein's stability\(^43\). Thus, miR-181a can be both a bad and a good diagnostic marker in HER2\(^+\) samples.

Our findings are not consistent with literature data on miR-25 overexpression in HER2\(^+\) IBC samples\(^5\).

**MiR-181a and miR-25 in TNBC samples**

TNBC is the most aggressive IBC subtype and is associated with rapid progression and poor prognosis\(^2\). We revealed increased miR-181a expression in the TNBC group compared to the potentially healthy controls, consistently with some literature data\(^8,^{10}\) and in contradiction to other findings\(^9\).

In basal-like tumours, the extent of genome methylation is the lowest among IBC cases\(^39\). The effect of miRNA in the TNBC group is unlikely to be mediated by methylation. It can be assumed that miR-181a acts as a TGF-\(\beta\)–regulated tumour progression regulator\(^{14,44}\). Genes related to the TGF-\(\beta\) signalling pathway are thought to be prognostic markers of TNBC\(^45\), whereas TGF-\(\beta1\) expression is known to be elevated in TNBC tissues\(^46\). Nonetheless, at an early stage of cancer progression, TGF\(\beta\) works as a tumour suppressor and promotes oncogenesis only at a later stage\(^44\).

Therefore, the observed overexpression of miR-181a in TNBC samples may indicate an increase in proto-oncogenic or tumour-suppressive transduction of the TGF-\(\beta\) signal or changes in other molecular pathways such as suppression of the pro-apoptotic BAX gene\(^47\).

Our findings are in line with literature data about miR-25, which is highly active in TNBC\(^20\). MiR-181a and miR-25 have been identified as components of an expression signature consisting of six miRNAs; this signature predicts the status of BRCA1/2 mutations, for which a standard test can give a false negative result\(^48\). Our results support the thesis that increased miR-181a and miR-25 expression may be an indication for more thorough testing for possible BRCA1/2 mutations\(^48\).

**MiR-181 in benign-breast-disease samples**

We revealed greater miR-181a expression in the fibroadenoma group \((p = 0.018537)\) and fibrocystic disease/adenosis group \((p = 0.029736)\) (a low risk of malignant transformation) as compared to the potentially healthy controls (Fig. 1). By contrast, miR-181a expression in the group with benign breast diseases having a high risk of malignant transformation did not differ from the control level.

Fibrous aberrations in breast tissue and simple cysts do not raise the risk of BC\(^49\), and fibrocystic breast disease is generally not associated with an increased risk of malignancy. Nonetheless, in certain histopathological and clinical conditions, fibrocystic disease may correlate with a BC risk (up to 50%)\(^50\).

The average age at diagnosis of carcinoma developing from fibroadenoma is 42.5 years, i.e., \(~20\) years after the most likely age at diagnosis of fibroadenoma. Therefore, malignant transformation should be
suspected in older women with fibroadenoma and a family history of BC and/or BRCA-1/2 mutations\textsuperscript{30}.

Very few data are available on the types of cancer that are more likely to arise via malignant transformation of benign breast diseases. It is known that microglandular adenosis can be a precursor to ER\textsuperscript{−} cancer\textsuperscript{2}. It is possible that changes in miR-181a and miR-25 levels can be useful in complex diagnostic protocols and for risk assessment of malignant transformation in benign breast diseases.

\section*{Conclusions}

We revealed elevated miR-181a expression levels in patients with a benign breast disease having a low risk of malignant transformation. Clinically significant molecular/genetic abnormalities have not been found in these patients\textsuperscript{2}, and this miRNA's level may be a negative predictor of precancerous changes.

In patients with IBC, miR-181a and miR-25 expression levels are generally higher in more malignant IBC subtypes (such as luminal B-like [HER2\textsuperscript{−}] and TNBC); however, this overexpression may reflect either favourable or adverse processes in the tumour owing to the multiple effects of the miRNAs in question, especially miR-181a.

MiR-181a and miR-25 can target mRNAs of several genes and simultaneously affect several targets, pathways and processes, including those that act differently in relation to tumour progression. These targets may be modulated by other miRNAs, thus pointing to the existence of autoregulatory loops. The use of miR-181a and miR-25 as markers seems to make sense only in a set of other markers. It is feasible to develop a diagnostic panel that would include both miR-181a and miR-25 and their target genes.

\section*{Declarations}

\subsection*{Competing interests}

The authors declare no competing interests.

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\subsection*{Author contributions}
M.P. performed the experiments and data analyses and wrote the first draft of the manuscript; A.S. performed the experiments and data analyses; A.P. contributed to the data collection and management; A.G. participated in project administration and in interpretation of the data; A.A. conceived and designed the study and participated in interpretation and analysis of the data and in project administration. All authors have reviewed and approved the manuscript.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

References


Figures
Figure 1

Levels of miR-181a in serum samples from patients with benign breast disease or IBC and healthy controls. *p < 0.05.
Figure 2

Levels of miR-25 in serum samples from patients with a benign breast disease or IBC and healthy controls. *p < 0.05.