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Challenges of *in situ* hybridization in miRNA analysis of triple-negative breast cancer morphological diversity

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miRNA expression in triple-negative breast cancers (TNBC) has mainly been studied from a methodological viewpoint. However, it has not been considered that miRNA expression profile may be associated with a specific morphological entity inside every tumor. The verification of this hypothesis on a set of 25 TNBCs was the subject of our previous work, where we confirmed specific expression of the studied miRNAs in 82 samples of different morphologies including inflammatory infiltrate, spindle cell, clear cell, and metastases after RNA extraction and purification as well as microchip and biostatistical analysis. In the current work, we demonstrate a low suitability of in situ hybridization method for miRNA detection compared to RT-qPCR, and in detail discuss the biological role of 8 miRNAs with the most significant changes of expression.

Key words: triple-negative breast cancer (TNBC); miRNA; in situ hybridization; RT-qPCR

miRNAs (miRs) are noncoding molecules of RNA with a length of 18 to 25 nucleotides, which bind to the 3' end of inactive sites of "messenger" RNA (mRNA). This leads to the degradation of the respective mRNA and inhibition of the production of target proteins. In this way, miRNAs control the activity of over 50 % of human genes and regulate basic cellular processes at a posttranslational level. Their defective regulation and involvement in carcinogenic processes or developing resistance to treatment among others through the epithelial-mesenchymal transition (EMT) process has been established in many types of tumors including triple-negative breast cancers (TNBC) [1–15].

TNBC are a morphologically and genetically heterogeneous group of breast cancers characterized by a low or zero expression of estrogen and progesterone receptors, HER2/neu/ERBB2 protein, aggressive biological behavior, and poor response to treatment. To date, the heterogeneity of miRNA expression in TNBC has mainly been studied from a methodological viewpoint and it has not been considered, that it may be associated with a specific differentiation bound to a certain morphological entity inside every tumor. We, therefore, focused to verify this hypothesis on a set of TNBCs.

Patients and methods

We performed morphological retyping on 25 TNBCs, in which we subsequently performed miRNA expression profile analysis. Using laser microdissection (PALM RoboSoftware version 4.6, Carl Zeiss Microscopy GmbH, Germany), samples were collected from areas with differing morphology from paraffin-embedded tissues. Thus, we acquired a total of 82 tissue samples, which, after RNA extraction and purification, underwent microchip and biostatistical analysis followed by the analysis of expression in tissues using ISH and RT-qPCR. The obtained results were then verified on a validation set of 82 TNBCs using RT-qPCR. The selected miRNAs were then discussed with regard to their recent literary data.

Patient sets, tumor specifications, and sample processing. Methods of sample collection, processing, and evaluation, including the selection of the studied morphologies in TNBC, were described in detail in our previous work [1]. Here is a short summary: To analyze the expression of 2,578 human mature miRNAs in individual morphologies inside each tumor and surrounding non-tumorous tissue of the mammary gland, we randomly selected 25 TNBCs

diagnosed at the Department of Clinical and Molecular Pathology between the years 2010 and 2017 from patients, who did not undergo neoadjuvant chemotherapy and were primarily treated by surgery (in order to examine the entire tumor with preserved tumor morphology, undamaged by chemotherapy). We reviewed the histological tumor type and simultaneously identified various tumor morphologies inside each tumor. Isolation of whole RNA, including short miRNA molecules, for microchip and statistical analyses, was performed from samples obtained by microdissection from various areas of the tumor with differing morphologies (clear cell/apocrine, spindle cell, carcinoma *in situ* – CIS, areas with dense lymphocytic infiltration). Control samples were also obtained from the epithelial component of lobules or ducts from surrounding non-tumorous mammary gland tissue.

miRNAs detection by ISH. The isolated RNA was analyzed using microchip technology and the selected candidate miRNA, which best discriminated the individual morphologies within the tumor (changes in miRNA expression of at least 1.0 compared to normal mammary gland lobules were considered significant), were visualized by *in situ* hybridization (ISH) using miRCURY™ LNA™ microRNA ISH Detection Probes & Kit (Exiqon/Qiagen; Hilden, Germany) with specific „double DIG-labeled LNA" probes [1]. The manufacturer's protocol was slightly modified. To optimize the method, the duration of ISH was extended from the initially recommended 5 min at 60 °C temperature to 15 h at 37 °C temperature. The sections were washed in SSC solution at 50 °C (anti-DIG reagent; diluted 1:200). Incubation of the samples in AP substrate lasted 2 h at a temperature of 37 °C. The final steps of the procedure were in accordance with the manufacturer's protocol. The results extracted from our previous study are shown in Tables 1 and 2 [1].

In total, all 82 samples obtained from 25 tumors used for RNA isolation were examined by the ISH method, repeatedly 2–3 times in cases with non-standard and negative results. Satisfactory visualization of the expression was achieved only in part of the analyzed samples of 6 miRNAs (miRNA 143-3p, 150-5p, 185-5p, 200c-3p, 205-5p, 4417-5p). The expression of miRNAs 155-5p and 182-5p could not be visualized even once.

Results and discussion

Selection of candidate miRNAs for ISH based on paired and unpaired analysis of microchip detection results. Eight miRNAs, whose expression differed the most within the studied morphologies were selected for ISH from earlier published data concerning EMT [1]. These were miRNAs with decreased (143-3p and 205-5p) or increased expression (182-5p, 185-5p, and 4417), especially in the clear cell and spindle cell morphologies and miRNAs with significant changes in the lymphocytic infiltrate (decreased 200c-3p and increased 150-5p and 155-5p).

miRNA 143-3p. We demonstrated a strong decrease in the expression of all tumor morphologies, including CIS and areas with intense lymphocytic infiltrate, compared to the normal epithelium (Table 1 and Table 2). This fact was also confirmed using ISH (Figures 1A, 1B).

According to the published data, miRNA 143-3p acts as a tumor suppressor and is important in the development of resistance to chemotherapy in TNBC. In breast cancer cells, a decrease in its expression has been described and, by contrast, stimulation of its expression led to inhibition of proliferation and migration as well as induction of apoptosis [16]. Inhibition of miRNA 143 caused an increase in the expression of apoptosis inhibitor "protein cytokine-induced apoptosis inhibitor 1" (CIAPIN1) and activated MDR, contrarily the stimulation of expression increased sensitivity to chemotherapeutic agents. Therefore it is probable, that this tumor suppressor microRNA may be a new molecular target in achieving an effective reversal of chemoresistance in TNBC [17]. Other target proteins of this miRNA are MAPK7, MYBL2, LIMK1 [16–18]. In our analyzed set, expression was strongly decreased in all tumorous morphologies including CIS and areas with dense lymphocytic infiltrate compared to the normal epithelium. In this regard, our results are in accordance with previously published data.

miRNA 150-5p. We discovered that it discriminates areas with a high density of lymphocytes (increased expression) from all tumor morphologies, as well as the normal tissue (Tables 1 and 2). This fact was also confirmed using ISH (Figure 1C).

According to the literature, miRNA 150-5p is a typical tumor suppressor [19, 20]. It inhibits metastasis by affecting HMGA2 (directly acts on mRNA). It is inhibited in tumor tissue compared to surrounding normal mammary tissue. It is increasingly expressed in the invasive tumor component compared to the CIS component and reflects the positivity of ALDH in mammary cells. However, Huang et al. [21] reported that miRNA 150 is excessively expressed in tissues and cell lines derived from breast cancer. Inhibition of its action leads to cell death; however, ectopic expression leads to increased cell proliferation. Its effect is explained by its action on the pro-apoptotic purinergic receptor P2X7. Jia et al. [22] described its cooperation with long noncoding RNA (lncRNA) MAF BZIP transcription factor G antisense RNA 1 (MAFG-AS1), which is strongly upregulated in breast cancer. High MAFG-AS1 expression promotes the proliferation, migration, and epithelial-mesenchymal transition of breast cancer cells and serves as a sponge of miRNA 150-5p, and that miRNA 150-5p bound to MYB. According to our findings, expression of this miRNA discriminated areas with a high density of lymphocytes, where it was upregulated in comparison to all tumorous morphologies and normal tissue. Its expression in metastases to lymph nodes was not established. This result confirms the possible role of this miRNA in the suppression of tumor growth but at the same time supports the contemplation that its effect occurs because

Table 1. Unpaired analysis of miRNAs with the most significant difference in expression of the studied morphologies [1].

	apocrine	CIS	invasive	spindle	lymph	Ave Expr	F	p-value	adj. p-value
miRNA 143-3p	**–3.2**	**–4.2**	**–2.7**	**–2.1**	**–4.8**	4.0	11.7	9.09 E–08	5.86 E-05
miRNA 150-5p	0.0	–0.3	0.1	1.0	*5.3*	2.5	16.5	5.30 E–10	1.37 E-06
miRNA 155-5p	1.7	0.4	0.7	*3.3*	*4.5*	2.8	6.8	4.89 E–05	0.009691
miRNA 182-5p	*3.3*	1.4	*2.2*	1.6	–1.0	3.5	5.9	0.000181	0.021326
miRNA 185-5p	*2.1*	0.6	0.9	*2.5*	0.3	3.3	5.3	0.00048	0.031735
miRNA 200c-3p	1.1	0.7	0.8	–1.8	**–5.9**	8.4	14.1	5.89 E–09	7.59 E-06
miRNA 205-5p	**–4.0**	**–3.9**	**–3.2**	**–4.3**	**–6.2**	4.7	9.5	1.23 E–06	0.00053
miRNA 4417-5p	*2.9*	–0.5	0.5	0.2	0.0	2.8	7.7	1.35 E–05	0.003481

Notes: apocrine-apocrine/clear cell TNBC morphology; spindle-spindle cell TNBC morphology; Lymph-areas of TNBC with dense lymphocytic infiltration; invasive-invasive margins of TNBC; Abbreviations: CIS-carcinoma *in situ*; Ave Expr-average expression; F-F-value; adj. p-value-adjusted p-value

Table 2. Paired analysis of miRNAs with the most significant difference in expression of the studied morphologies [1].

	apocrine	CIS	invasive	spindle	lymph	Ave Expr	F	p-value	adj. p-value
miRNA 143-3p	**–3.1**	**–4.1**	**–2.8**	**–2.0**	**–4.7**	4.0	11.8	8.03 E-08	6.06 E-05
miRNA 150-5p	0.0	–0.2	0.1	1.0	*5.3*	2.5	16.7	4.04 E-10	1.04 E-06
miRNA 155-5p	1.8	0.5	0.7	*3.3*	*4.4*	2.8	6.8	4.73 E-07	0.009371
miRNA 182-5p	*3.3*	1.6	*2.2*	1.5	–1.0	3.5	6.0	0.000151	0.018557
miRNA 185-5p	*2.1*	0.7	0.9	*2.5*	0.3	3.3	5.4	0.000424	0.029571
miRNA 200c-3p	1.1	0.7	0.8	–1.8	**–5.9**	8.4	14.2	5.43 E-09	7.00 E-06
miRNA 205-5p	**–3.8**	**–3.8**	**–3.2**	**–4.3**	**–6.1**	4.7	9.6	1.15 E-06	0.000511
miRNA 4417-5p	*2.8*	–0.4	0.5	0.2	0.0	2.8	7.5	1.75 E-05	0.00422

Notes: apocrine-apocrine/clear cell TNBC morphology; spindle-spindle cell TNBC morphology; Lymph-areas of TNBC with dense lymphocytic infiltration; invasive-invasive margins of TNBC; Abbreviations: CIS-carcinoma *in situ*; Ave Expr-average expression; F-F-value; adj. p-value-adjusted p-value

of processes associated with inflammation and the immune reaction. There were no significant differences in expression between CIS, tumorous morphologies, and normal lobules of the mammary gland in our set.

miRNA 155-5p. We found that it discriminates areas with a high density of lymphocytes, including spindle cell morphology, from other morphologies (Tables 1 and 2).

miRNA 155-5p expression levels have been found to be upregulated and serve as a negative prognostic marker in numerous types of solid cancer, including human breast cancer. Its expression positively correlated with poor prognosis of numerous types of tumors and TNBC resistance to cetuximab and paclitaxel [23, 24]. TP53INP1 was identified as a direct target gene of miRNA 155-5p. miRNA 155-5p is probably a key oncogenic microRNA, which ensures immune homeostasis and mediates the coordination between inflammation and carcinogenesis [25–28]. Expression is typical for one of the "basal-like" subtypes of breast cancer. Its expression protects from the apoptotic effect of certain toxins. According to other authors, it functions as a tumor suppressor, which is inhibited in breast cancers. However, this controversial finding is rare.

In our set, this miRNA was increasingly expressed, similarly to miRNA 150, in areas with high lymphocytic density, which discriminated it from other morphologies. However, it was also increasingly expressed in morphologies typical for EMT in TNBC (spindle cell and clear cell component), which does not rule out the possibility, that these morphologies were contaminated by lymphocytic infiltrate.

miRNA 182-5p. We discovered increased expression in all tumor morphologies, compared to areas with dense lymphocytic infiltrate, where it was decreased (Tables 1 and 2).

miRNA 182-5p acts as an oncogenic miRNA causing greater proliferation, migration, and tumorigenesis of BC, which is "upregulated" in most BCs [29–33]. Increased levels are seen in TNBC. It regulates sensitivity to tamoxifen [31] and may indicate sensitivity to chemotherapy. It affects the network of genes associated with DNA repair and potentiates metastases of "basal-like breast cancer" [32]. miRNA 182-5p was highly expressed in breast cancer tissues and cells, and this high expression was associated with poor prognosis of breast cancer patients, its overexpression was shown to promote tumor angiogenesis in breast cancer. Lu et al. [33] found that CMTM7 is a target of miRNA 182-5p as together they promote tumorigenesis and metastasis of breast cancer cells by regulating the CMTM7/EGFR/AKT signaling axis.

In our set, we demonstrated its upregulation in all tumorous morphologies compared to areas of dense lymphocytic infiltrate as well as normal mammary gland structures, in which it was decreased. This result supports previously published data regarding the oncogenic effect of this miRNA.

miRNA 185-5p. We described that it discriminates clear cell and spindle cell morphologies from other morphologies, as well as from normal tissue. Expression was also

seen in metastases to the lymph nodes, but also in CIS with a representative clear cell component (Tables 1 and 2; Figures 1D, 1E).

To date, miRNA 185-5p was considered to be a tumor-suppressing RNA [34, 35]. Its expression is said to be inversely associated with metastasis to the lymph nodes, clinical stage, overall survival, and symptom-free interval, and its ectopic expression to inhibit proliferation *in vitro* and *in vivo*. Shi and co-workers [35] described a decreased expression in TNBC, lymph node metastases, and tumors with worse

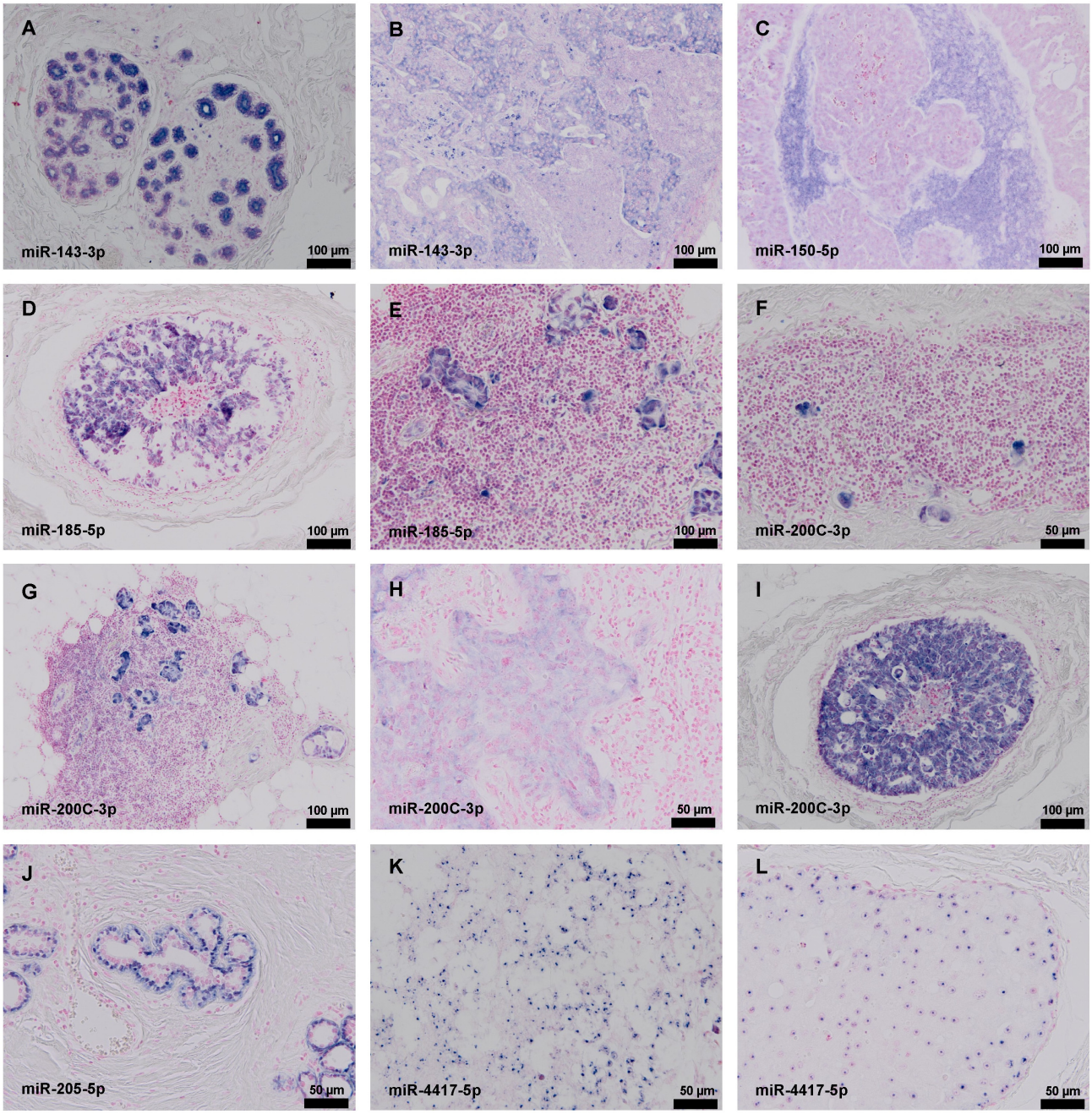

Figure 1. A, B) Normal lobules of the mammary gland (A) with significantly higher expression of miR 143-3p than in the tumor cells (B). C) Positivity of miRNA 150-5p in the lymph node and negativity in the tumor cells. D, E) miRNA 185-5p positivity in CIS (D) and in metastasis to the lymph node (E). F, G) Positivity of miRNA 200c-3p in tumor micrometastases to the lymph nodes (there is no increased expression, but rather a difference between expression in tumor cells and lymph node lymphocytes, in which there is practically zero expression). H, I) Decreased expression of miRNA 200c-3p in invasive tumors (H) compared to the CIS component (I). J) Positivity of miRNA 205-5p in myoepithelial cells and negativity in lymphocytes. K, L) Increased expression of miRNA 4417-5p in the nucleoli in areas of clear cell morphology with apocrine transformation.

prognostic parameters. We recorded increased expression in metastasizing tumors; however, we must consider that there were only 4 cases of metastases to the lymph nodes in our set.

Based on all our results, we can state that expression of this miRNA, similarly to miRNA 182, was "upregulated" in tumor morphologies compared to normal structures or lymphocytic infiltrate, and thus we consider the suppressor role of this miRNA in TNBC debatable.

miRNA 200c-3p. In our set, its expression was decreased in the lymphocytic infiltrates, less significantly in the spindle cell morphology. It discriminates the lymphocytic background and partly the spindle cell component from other morphologies. It was established in micrometastases of TNBC to the lymph nodes using ISH. In certain cases, reduced expression was observed in the tumor compared to CIS (Tables 1 and 2; Figures 1F–1I).

The literature reports that miRNA 200c-3p acts as a tumor suppressor in TNBC and may be a key miRNA in the development of breast cancer [36–43]. It is involved in the regulatory axis p53/miRNA 30a/ZEB2, which controls the invasiveness and further spreading of tumor cells. It is a tumor suppressor miRNA associated with inflammation and plays an anti-inflammatory role. It may affect inflammatory infiltration, inhibit the production of IL-6, IL-8, CCL-5, and is involved in the pathways affecting inflammation. In TNBC, a reduction in the expression of miRNA 200c, similarly to miRNA 205, enables the prediction of metastasis to the lymph nodes [44], or this miRNA may inhibit metastasizing by affecting the regulator of epithelial-mesenchymal transition ZEB2. Furthermore, it was discovered, using the qPCR method, that miRNA 200c levels are significantly lower in TNBC compared to the normal adjacent tissue. Also, "basal-like" subtypes of breast cancer had lower miRNA 200 levels than luminal subtypes, and patients with decreased miRNA 200c expression exhibited a lower response to chemotherapy, while increased expression sensitized to epirubicin, partly by regulating the MDR-1 gene associated with expression of P-glycoprotein. miRNA 200c also increased the sensitivity of TNBC cells to undergo "anoikis" by affecting NF-κB and increasing the activity of "tropomyosin receptor kinase B/neutrophin 3" of the autocrine signaling pathway. Increased expression by members of the miRNA 200 family was typical for the epithelial phenotype of TNBC, while excessive expression of miRNA 221/222 led to the development of the "low-grade mesenchymal-like" phenotype of TNBC.

Our study established its significant "downregulation", especially in areas with intense lymphocytic infiltration of TNBC. Other morphologies did not exhibit substantial changes in expression compared to normal mammary gland lobules. Using ISH and microchip analysis, a reduction in expression in even the spindle cell component of the tumor was observed, compared to other morphologies. This decrease may have been associated with lymphocytic infiltration in this area. The difference in expression between the lymphocytic and tumorous components may explain its higher visualization in micrometastases to the lymph nodes.

Our results support the involvement of this miRNA in processes regulating the inflammatory response and immunity. We established its "downregulation" in areas with dense lymphocytic infiltrate, through which it discriminates the lymphocytic background and partially the spindle cell component. We believe, that in the above-mentioned publications, the presented "downregulation" of this miRNA in TNBC was not directly associated with changes in the expression of tumor cells, but rather in the lymphocytic infiltrate, which is often very intense, especially in this subtype of breast cancer.

miRNA 205-5p. We demonstrated that it discriminates tumorous morphologies as well as lymphocytic infiltrate (where expression is strongly decreased), compared to the normal tissue, and was also insignificantly decreased in CIS (Tables 1 and 2). Expression was observed in the myoepithelial component of normal lobules (Figure 1J) and ducts.

miRNA-205-5p may function as a tumor suppressor. Its decreased expression is strongly associated with poor prognosis and shorter symptom-free interval [44–50]. Along with „long non-coding RNA FGF14-AS2", it suppresses proliferation, migration, invasiveness, and induces apoptosis in breast cancer. Reduced expression correlates with the metastatic potential of breast tumors.

miRNA-205 is one of the most studied miRNAs and existing research confirms its involvement in numerous physiological, as well as oncogenic or tumor-suppressor, regulatory pathways. A regulatory malfunction at the transcription level has been demonstrated primarily in tumors, depending on the tumor type. Epigenetic modification of the mutated or alternatively spliced protein p53 and other members of this protein family, as well as the tumor microenvironment (hypoxia, proinflammatory cytokines), play a role. Post-transcriptionally, lncRNAs are responsible for changes in miRNA 205 availability for tumor cells. The situation is further complicated by the proximity to the promoter of the gene determining its biosynthesis during genome rearrangement. In the mammary gland, this miRNA is highly expressed in the basal myoepithelial layer of lobules and ducts. Its excessive expression leads to the expansion of progenitor cells, reduction in cell size, and increased cell proliferation. This effect is mediated by suppressing the expression of the tumor suppressor PTEN (phosphatase and tensin homolog). Expression of miRNA 205 is also increased during gestation and late post-gestational involution. It has been generally accepted, that regulation of miRNA 205 is necessary for the development of diverse types of epithelia. Alteration of its normal expression was observed during the initiation and progression of numerous tumors of epithelial origin. There is increasing evidence to support the heterogeneity of its expression inside certain tumor type. For example, its excessive expression is typical for the squamous cell phenotype in NSCLC or esophageal carcinomas and decreased expression

for the glandular phenotype. These results indicate the dual role of miRNA 205 in various tumor types and raise interest in upstream regulators of its expression.

In our set of TNBCs, we observed its significant downregulation in all tumorous morphologies, but mostly in the lymphocytic infiltrate. This result confirms its significance in suppressing tumor development. However, its significant reduction in lymphocytic infiltrate also allows speculation regarding its role in inflammatory and immunity processes. Simultaneously, its expression in the basal myoepithelial component of normal lobules and ducts was confirmed. This result may validate an association between the expression of this miRNA and a „basal-like" phenotype of TNBC.

miRNA 4417-5p. Expression of this miRNA was the only one to differentiate clear cell morphology (where it was increasingly expressed) from other tumorous and non-tumorous morphologies (where its expression was comparable to the normal tissue). Expression was primarily observed in the nucleoli of tumor cells (Figures 1K, 1L).

miRNA 4417-5p also acts as a tumor suppressor, whose expression is suppressed during TNBC progression and is significantly lower in tumors with a worse prognosis. Expression is significantly more frequent in cancers with BRCA mutation [51, 52].

In our set, expression was increased in the clear cell/apocrine component of the tumor, thus significantly discriminating it from other tumorous and non-tumorous morphologies (where it was comparable to the normal tissue). This suggests the possibility of using this miRNA as a prognostic biomarker.

Comparison of ISH and RT-qPCR validation of presented miRNAs. Our experience with miRNA detection in these tumors using ISH was contradictory and the results were not standard. We demonstrated that quality results depicting a precise distribution of miRNA in tissues are only obtained sporadically because each step of the hybridization and visualization reaction requires the individual setting of the probe concentration, incubation duration, etc., depending on even slightly differently fixed and processed tissue samples. Due to anomalous visualization results, which were affected by the different behavior of each tissue, we simultaneously performed result validation of the microchip analysis using qRT-PCR on an extended set of 82 TNBCs. Although we could not use the ISH method for definitive confirmation of the hypothesis regarding the association between cell morphology and miRNA expression, it helped us to discover several interesting findings. Simultaneously, a very good concordance between microchip and RT-qPCR analysis was established, which confirmed the specific signature of miRNA expression in various morphologies of TNBC [1]. This method was more effective and less expensive.In conclusion, the results confirmed specific expression of the studied miRNAs in morphologies associated with inflammatory infiltrate/immune reaction, spindle cell and clear cell differentiation and metastases. Based on our presented results, a specific miRNA expression signature was determined for the following morphological entities. *Spindle cell tumor configuration:* decreased expression of miRNA 205, miRNA143, increased expression of miRNA 185. *Clear cell tumor configuration:* decreased expression of miRNA 205, miRNA 143, miRNA 145, increased expression of miRNA 182 and miRNA 4417. *Lymphocytic infiltrate:* decreased expression of miRNA 200 and miRNA 205, increased expression of miRNA 150 and miRNA 155. *Metastasis to lymph node:* lower expression of miRNA 150 (compared to the lymphocytes, where miRNA 150 expression is increased) and increased expression of miRNA 185 (in miRNA 200 there isn't an increased expression, but rather a difference in expression between tumor cells and lymph node lymphocytes, where it is almost zero).

The miRNA expression profile may reflect the state of metabolic or proliferative/regression activity and is somewhat of an accompanying feature of the situations taking place in TNBC during tumor progression or regression. Nonetheless, the association of these processes with morphological expressions is very probable.

Following priority results were determined in this study: a) expression of miRNA 200c in TNBC is strongly "downregulated" in inflammatory lymphocytic infiltrates, which are very frequent in these tumors, b) previously described "downregulation" of miRNA 200c expression isn't necessarily associated with changes in the expression of tumor cells, but may reflect the degree of infiltration of tumor tissue by tumor-infiltrating lymphocytes, c) as described previously [1], we confirmed the involvement of miRNA 4417 in the morphogenesis in tumor tissue and its "upregulation" in the clear cell/apocrine component of the tumor, d) we demonstrated low suitability for miRNA detection using the ISH method compared to RT-qPCR for validation purposes in larger cohort samples.

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