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ORIGINAL ARTICLE

miR-301a-5p negatively affects Treg and Th17 related gene expression in contrast to miR-21 and miR-23b-5p in early-stage breast tumor tissue

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Summary

Purpose: *To study local levels of 5p-arms of microRNA (miR) miR-23b, miR-301a, and miR-21 in relation to T regulatory (Treg)/T helper 17 (Th17) related genes expression in paired tissue samples of breast cancer (BC) patients and its relationship with clinical characteristics of BC.*

Methods: *Fresh primary tumor samples and adjacent normal (non-tumoral) tissue specimens were used for miRs and total RNAs isolation. The synthesis of cDNA was performed by reverse-transcription PCR. The relative quantity of miR-21, miR-23b-5p, and miR-301a-5p as well as of eight Treg/Th17 related target genes were determined by TaqMan based qPCR in paired tissue samples of early stages, luminal BC and their normal counterparts. Relative quantification analysis was performed by the comparative Ct (Cq) method.*

Results: *The mean level of miR-21 was significantly upregulated over 6-fold (p=0.002) in tumoral tissue, while the levels of miR-23b-5p and miR-301a-5p were significantly higher in larger tumors (T1 vs. T2: p=0.014; p=0.009, respectively). The level of miR-301a-5p was in strong inverse relation with TGFBR2 (r=-0.6668; p=0.0035) and IL10 (r=-0.6356; p=0.0061). On the contrary, miR-21 was in positive relation with TGFBR2 (r=0.592; p=0.01); TGFB1 (r=0.6308; p=0.0066); IL10 and FOXP3 with borderline significance. Furthermore, miR-21 was positively correlated with IL6 gene expression (r=0.5669; p=0.018), RORC (r=0.5024; p=0.039) and IL12B (r=0.8999; p=0.0374).*

Conclusions: *Our study confirms the oncogenic role of miR-21 and suggests that its function is antagonized by miR-301a-5p as a part of the negative feedback mechanisms contributing to Th17-like Treg cancer-associated inflammation in the early-stage, luminal BC.*

Key words: *breast cancer, miR-21, microRNA, transforming growth factor beta receptor II*

Introduction

Since the discovery of micro RNAs (miRNA or MiRs), an increasing number of studies on their expression, biogenesis, and function in post-transcriptional regulation of target gene expression have been performed. Many miRs were found to be involved in carcinogenesis by modulating several processes including cell proliferation, differentiation, apoptosis, angiogenesis, migration, and control of the immune system responses [1]. Some of the known miRNAs were classified as oncomiRs or tumor suppressors, and plenty of conflicting reports exist [2].

Breast cancer (BC) is the most frequent malignancy in females in developed countries. Several studies investigate miRNAs levels in tissue and plasma of BC patients, or in cell lines and

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animal models aimed at clarifying the molecular mechanisms of carcinogenesis, identifying the new therapeutic targets or discovering new biomarkers for screening and monitoring [3]. Iorio et al [4] reported 29 significantly dysregulated miRNAs expression in BC tissues. Among them, miR-21 and miR-155 were upregulated and miR-10b, miR-125b, and miR-145 were down-regulated. The miR-21 upregulation in BC was also confirmed by other studies [5,6]. However, significant bias exists with respect to the relation between miR-21 and the characteristics of BC. Also, low miR-23b expression was correlated with BC metastasis [7], while miR-301a is pointed as oncomiR of which elevated expression indicates a poor prognosis for BC patients [8].

On the other hand, many reports demonstrated that miR-21, miR-23b, miR-301a and others are regulated by or regulating several immune-mediated mechanisms, critical for carcinogenesis, including the inflammatory response, the development, and activation of T helper 17 (Th17) and T regulatory cells (Treg) [9-12]. Several studies, including our previous studies, showed the significant contribution of Th17/Treg balance in the promotion and progression of different cancers [13-15].

In the human genome, most of the miRNAs are encoded by introns, such as the mir-21, mir-23b, and mir-301a, and are processed by canonic miRNA biogenesis [16,17]. It is largely accepted that one strand of the duplex, known as a guide strand, is responsible for mRNA-silencing, whereas the passenger strand, often marked as miRNA*, is degraded. However, previous studies reported that both stands (today labeled as 5p-arm and 3p-arm) of a particular miRNA could be functionally active [3,18,19] and suggesting that strand selection depends on cell type, disease stage and many other circumstances [20]. Currently, there are no available data about the role of miR-23b-5p and miR-301a-5p in breast carcinogenesis, although some studies reported their involvement in other cancers [21-23].

In respect to the above said, the aim of the present study was to investigate the involvement of miR-23b-5p, miR-301a-5p, and miR-21-5p in early stages luminal BC tissue samples. In addition, we explored their relationship with the local expression levels of Treg related genes, as transcription factor forkhead box P3 (*FOXP3*), transforming growth factor beta 1 (*TGFB1*), transforming growth factor, beta receptor II (*TGFBR2*), interleukin-10 (*IL10*) and Th17 related genes, as transcription factor RAR related orphan receptor C (*RORC*), the genes encoding the two subunits of IL-23, precisely interleukin-23p19 subunit (*IL23A*) and interleukin-12/23p40 subunit (*IL12B*), and interleukin-6 (*IL6*).

Table 1. The clinicopathological characteristics of the enrolled breast cancer patients

Characteristics	*Number of patients*
Age (years)	
Mean ± SD	65.55 ± 11.74
Median (min-max)	65.0 (46–85)
Premenopausal, n (%)	5 (25)
Postmenopausal, n (%)	15 (75)
Histological type , n (%)	
Ductal	17 (85)
Lobular	3 (15)
Clinical stage, n (%)	
1	3 (15)
2a	8 (40)
2b	9 (45)
Tumor size	
mean ± SD	2.15 ± 0.84
Median (25^{th}%-75^{th})	2.0 (1.5-2.5)
n (%)	
≤ 2 cm	6 (30)
>2 cm	14 (70)
T staging, n (%)	
T1	4 (20)
T2	16 (80)
N staging, n (%)	
N0	5 (25)
N1	15 (75)
Tumor subtype, n (%)	
Luminal A	8 (40)
Luminal B	12 (60)
ER status, n (%)	
Positive	17 (85)
Negative	3 (15)
PR status, n (%)	
Positive	15 (75)
Negative	5 (25)
HER2 status, n (%)	
Positive	6 (30)
Negative	14 (70)
Ki67	
mean ± SD	21.5±18.9
Median (25^{th}%-75^{th})	19.5 (5-26)
n (%)	
≤15	9 (45)
>15	11 (55)

ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2; SD: standard deviation

Methods

Study subjects

In the current study, adult female patients with early-stage, luminal BC were recruited from the oncology center and University Hospital "Professor doctor Stoyan Kirkovich" in Stara Zagora, Bulgaria. The study was approved by the ethics committee of the Medical Faculty, Trakia University (protocol no.4/25.04.2017). Written informed consent was obtained from each participant prior to enrolment in this study according to the ethical guidelines of the Helsinki Declaration. Fresh primary tumor samples (n=20) and adjacent normal (non-tumoral) tissue specimens (n=20) were collected during surgery. Non-tumoral tissue samples were obtained from a different quadrant after mastectomy or from normal breast tissue at least 2 cm around the tumor after quadrantectomy. All samples had been flash-frozen and stored at -80°C.

The tumor stage was classified according to the American Joint Committee on Cancer (AJCC) guidelines [24]. Patients with distant metastases, visualized by conventional or non-surgical approaches, were excluded. The mean age of included patients was 65.55±11.74 years (range, 46-85). All patients were untreated and unrelated Caucasian women. The expression of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and proliferating marker Ki67 was determined by standard immunohistochemistry methods. The tumor subtypes were defined according to well established molecular sub-classification in clinical use [25] as follows: Luminal A-like: tumors expressed ER and PR (ER+ and PR+) in addition to negative HER2– (HER2-) and low Ki67 (Ki67≤15%). The luminal B-like subtype comprised ER+ and/or PR+, HER- and high Ki67 (>15%) tumors or ER+ and/or PR+ and HER2+. The triple negative/basal like (ER-, PR-, and HER2-) and HER2 overexpressing tumors (ER-, PR- and HER2+) were excluded. The clinicopathological data of BC patients are summarized in Table 1.

Extraction of tissue RNAs and Reverse-transcription PCR

Isolation of miRNAs of paired tissue samples was performed by using the miRNA - mirVana™ miRNA Isolation kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

Reverse transcription (RT) was performed by the TaqMan Advanced miRNA cDNA synthesis Kit (Thermo Fisher Scientific, Inc.). RNA sample concentration, enriched for small species, was less than 5 ng/ml before adding to the reaction mixture for the poly (A) tailing reaction. After temperature incubation at 37°C for 45 min and 65°C for 10 min, the adaptor ligation reaction was performed. The ligation product was converted to cDNA by next reaction using the universal RT primers. A preamplification step of cDNAs was performed at the following cycling parameters: 5 min at 95°C; 14 cycles of 3s at 95°C, 30s at 60°C, and final step of 10 min at 99°C. The PCR was run on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA).

Isolation of total RNA of paired tissue samples from the tumoral BC and non-tumoral mucosa was performed by using a GeneJet RNA purification kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The total RNA was quantified spectrophotometrically (GeneQuant 1300 spectrophotometer, GE Healthcare Life Sciences, Switzerland). Synthesis of cDNA was performed by using RevertAid First Strand cDNA Synthesis kit with random hexamers (Thermo Fisher Scientific, Inc.).

Quantitative real-time PCR

TaqMan quantitative real-time PCR of miRNAs was performed by using the miRNA-specific TaqMan™ Advanced miRNA assays kits (Applied Biosystems Life Technologies, CA, USA) as follows: miR-21-5p (assay ID: 477975_mir), miR-301a-5p (assay ID: 478796_mir), miR-23b-5p (assay ID: 477991_mir) and miR-520d-5p (assay ID: 478616_mir). The latter was used as normalizer, according to the reported high stably expressed levels in different biological matrices [26]. All samples were assessed in triplicate. The cycling conditions were as follows: initial hold of polymerase activation 20s at 95°C, followed by 40 cycles of denaturation 3 s at 95°C and annealing/extension 30s at 60°C.

Quantification of mRNAs of 8 target genes, *FOXP3, TGFBR2, TGFB1, IL10, IL6, RORC IL23A*, and *IL12B* was performed. The glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and 18S rRNA were used as reference genes. The used primers-probes were predesigned, inventoried and manufactured by Thermo Fisher Scientific Inc. The temperature conditions were according to the manufacturer's instruction, starting with initial denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15s and annealing extension step at 60°C for 60s.

The data were collected with 7500 Software v.2.3 (Life Technology, Foster City, CA, USA). Relative quantification was analyzed by the comparative Ct (Cq) method, also referred to as $2^{-\Delta\Delta Ct}$ method, described by Livak and Schmittgen [27]. The data are presented as fold change (relative quantity - RQ) of target genes relative to the non-tumoral tissue (calibrator) after normalization to the averaged reference genes. The final gene expression results were log-transformed to achieve a normal distribution of data and usage of parametric statistical tests. All quantitative real-time PCR reactions were run on a AB7500 Real Time System (Applied Biosystems, Life Technologies, USA).

Statistics

The normality of the data distribution was assessed by the Shapiro-Wilk test. The variables with normal distribution were analyzed using the parametric analysis Student's t-test or ANOVA, and the data are reported as mean±standard deviation (SD). Pearson's correlation analysis was performed to test the relationship between linearly related variables. In any case, the differences were considered significant with p values < 0.05. The statistical analysis was performed using the Statistica v. 12 (StatSoft, Inc., USA).

Results

The levels of miR-21, miR-301a and miR-23b in breast cancer tissues

The levels of miR-21, miR-301a-5p and miR-23b-5p were quantified in 40 paired tissue samples - tumoral and adjacent non-tumoral breast tissues. The individual relative expression of studied miRNAs in each patient is shown in Figure 1.

In most of the samples, miR-21 was upregulated, while significant inter-individual differences were observed for miR-301a-5p and miR-23b-5p. After stratification of individual data as upregulated over 1.5-fold change, downregulated under 0.66-fold, or unchanged in a range of change between 0.667-1.5, we observed that miR-21 expression was upregulated in 14 out of 20 tumoral samples (70%), miR-23b-5p in 9 (45%) and miR-301a-5p in 7 (35%).

Significantly higher mean levels of miR-21 was detected in tumoral tissues compared to their normal counterparts (Table 2). More than a 6-fold higher miR-21 level was observed in early-stages luminal BC tissue. There was no significant change in the mean value of miR-23b-5p and miR-301a-5p levels, although the individual data were heterogeneous.

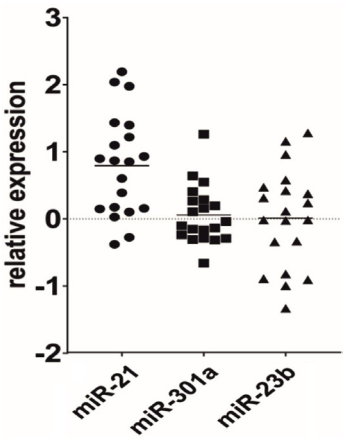

Figure 1. MiRs level in breast cancer. Dot plots show relative expression of the miR-21, miR-301a-5p, and miR-23b-5p in tumor tissue of breast cancer patients (n=20). The individual data are shown after calibration to the paired non-tumoral tissue and normalized to the reference miR-520d. The relative expression was log-transformed.

Association of miRs levels and clinicopathological characteristics of breast cancer

The associations between miRs levels and clinicopathological characteristics of BC are shown in Table 3. The obtained results indicate that higher miR-23b-5p and miR-301a-5p levels were significantly associated with T2 cancers (p=0.014 and p=0.009, respectively). MiR-21 and miR-23b-5p expression in primary tumor tissue were significantly higher in node-negative BC than BC with 1–3 positive lymph nodes (p=0.0019 and p=0.025, respectively). MiR-21 levels were higher in ER+, PR+, HER- and higher proliferation index (Ki67>15) without reaching statistical significance. In addition, there was a trend for upregulated miR-23b-5p in ER+ and PR+ than negative BC. No other significant association was found between expression of the studied miRs and receptors status, proliferating activity, clinical subtype (luminal A and B), and BC stage (stage 1, stage 2a, and stage 2b).

Correlation between miRs and Treg related genes - FOXP3, TGFB1, TGFBR2 and IL10 mRNA levels in breast cancer tissues

The correlation analysis between expression of miRNAs and Treg related gene is shown in Figure 2.

All studied Treg related genes were inversely correlated with miR-301a-5p and positively correlated to miR-21. Significantly strong inversely relation was detected between miR-301a-5p and *TGFBR2* (r=-0.6668; p=0.0035) and *IL10* (r=-0.6356; p=0.0061). In contrast, miR-21 was in positive correlation with *TGFB1* (r=0.6308; p=0.0066), *TGFBR2* (r=0.592; p=0.01), *IL10* and *Foxp3* with borderline significance. No significant correlations were detected between miR-23b and Treg related genes.

Table 2. Relative quantity of miR-21, miR-301a-5p and miR-23b-5p in breast tumoral and non-tumoral (normal) tissue specimens

Target	*ΔCt Non-Tumoral tissue*	*ΔCt Tumoral tissue*	*ΔΔCt*	*Fold of change ($2^{-\Delta\Delta Ct}$ ± SD)*	*p value*
miR-21	-8.447	-11.038	-2.591	**6.025±4.86**	**0.002**
miR-301a-5p	-1.835	-2.032	-0.197	1.146±2.74	0.696
miR-23b-5p	1.117	1.085	-0.032	1.022±5.7	0.965

miR = microRNA; $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{reference gene}}$; $\Delta\Delta Ct = \Delta Ct_{\text{tumoral tissue}} - \Delta Ct_{\text{non-tumoral tissue}}$; SD - standard deviation
The statistical significant values are presented in bold.

Correlation between miRs and Th17 related genes - RORC, IL6, IL23A, and IL12B mRNA levels in breast cancer tissues

Correlation analysis between expression of miRNAs and Th17 related gene is shown in Figure 3.

Significantly positive moderate correlations between miR-21 and Th17 specific transcription factor *RORC* (r=0.5024; p=0.04) were detected. Also, miR-21 and miR-23b-5p were positively correlated with *IL6* gene expression (r=0.5669; p=0.018 and r=0.4539; p=0.067, respectively) in contrast to miR-301a-5p (r=-0.4796; p=0.051). The expression of *IL12B* was in strong negative correlation with miR-301a-5p (r=-0.9866; p=0.0019) and positively correlated with miR-21 (r=0.8999; p=0.037) and miR-23b-5p (r=0.9453; p=0.015). Of note, *IL12B* expression was not detected in all samples and these data should be considered with caution.

Discussion

The most significant result of our current study is that miR-23b-5p and miR-301a-5p upregulation was significantly associated with larger tumor size in early stages BC. MiR-301a-5p expression was in inverse correlation to *TGFBR2, IL10* and *IL12B* gene expression in contrast to miR-21. These data

Table 3. MiRNAs expression and clinicopathological characteristics of the breast cancer

Target/characteristics	*Fold of change ($2^{-\Delta\Delta Ct}$ ± SD)*			*p value*
Stage	*Stage 1*	*Stage 2a*	*Stage 2b*	
miR-21	10.813 ± 19.14	6.745 ± 4.29	5.088 ± 5.35	0.713
miR-301a-5p	0.735 ± 1.29	0.832 ± 2.52	1.508 ± 2.76	0.630
miR-23b-5p	0.151 ±1.12	1.449 ± 6.40	1.205 ± 4.83	0.544
T	*T1*	*T2*		
miR-21	3.058 ± 4.34	4.707 ± 10.48		0.596
miR-301a-5p	0.516 ± 1.67	1.370 ± 2.85		**0.009**
miR-23b-5p	0.314 ± 3.44	1.569 ± 5.97		**0.014**
N	*N0*	*N1*		
miR-21	22.419 ± 1.3	3.152 ± 4.1		**0.0019**
miR-301a-5p	1.167 ±3.19	0.931 ± 2.97		0.313
miR-23b-5p	2.224 ±11.7	0.681 ± 4.42		**0.025**
ER status	*positive*	*negative*		
miR-21	6.054 ± 5.05	1.029 ± 21.66		0.268
miR-301a-5p	0.958 ± 1.99	1.679 ± 6.36		0.432
miR-23b-5p	1.387 ± 4.7	0.319 ± 19.07		0.276
PR status	*positive*	*negative*		
miR-21	4.931 ± 3.87	4.427 ± 16.9		0.928
miR-301a-5p	0.889 ±2.0	1.874 ± 7.23		0.325
miR-23b-5p	1.505 ± 3.88	0.407 ± 22.4		0.312
HER2 status	*positive*	*negative*		
miR-21	4.038 ± 4.38	7.630 ± 6.2		0.569
miR-301a-5p	1.073 ± 2.64	0.897 ± 1.8		0.708
miR-23b-5p	1.964 ± 4.99	1.137 ± 5.29		0.609
Ki67	*<15*	*>15*		
miR-21	3.179 ± 2.69	5.898 ± 7.05		0.571
miR-301a-5p	0.899 ± 1.52	1.169 ± 3.75		0.713
miR-23b-5p	1.454 ± 1.63	0.938 ± 9.86		0.719
Luminal	*A*	*B*		
miR-21	6.880 ± 9.4	5.627 ± 4.21		0.858
miR-301a-5p	1.163 ± 1.95	0.857 ± 2.12		0.518
miR-23b-5p	2.549 ± 2.56	0.980 ± 6.26		0.363

ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2; SD: standard deviation
The significant values are presented in bold.

suggested that 5p-arms of miR-23b and miR-301a also have functional activities that could be utilized in the processes of breast carcinogenesis.

As expected, we observed a significantly higher miR-21 level (more than 6-fold) in BC tissue than in their normal counterparts. In 70% of tumoral tissues, miR-21 was upregulated more than 1.5-fold. Several earlier studies reported significantly upregulated miR-21 in BC tissue [4-6], however, there are considerable divergences of reported results with respect to miR-21 correlation with clinicopathological characteristics of BC. Our study showed higher miR-21 expression in lymph node negative BC ($p<0.001$), very similar miR-21 levels between BC stages (stages 1, 2a and 2b), tumor size (T1 and T2 status), and luminal A and B subtypes. Elevation of miR-21 was observed in ER+, PR+, HER- and higher proliferation index (Ki67>15) BC, without reaching statistical significance. Similar data were reported previously in matched formalin-fixed paraffin-embedded tissues and frozen tissue samples of 113 patients with BC [5]. Our finding of high miR-21 expression in early stage luminal BC confirms its undeniable role in carcinogenesis and suggests that miR-21 affects critical molecular events involved in BC promotion, evident even in early stages of BC.

On the other hand, it has been reported that miR-21 contributes to the imbalance of Th17 and Treg cells in autoimmunity and is involved in the early and late inflammatory response [9, 12]. The regulation of miR-21 transcription has been reported to be triggered by many pro-inflammatory stimuli under control of NF-kB, STAT3, and AP-1 [28]. In addition, there are pieces of evidence that miR-21 expression could be driven by TGF-β signaling that is also involved in the processing of pri-miR-21 [17]. Our data show significant positive correlation of miR-21 with *TGFB1* (r=0.63; p=0.007), *TGFBR2* (r=0.59; p=0.01), *IL6* (r=0.57; p=0.02), *IL12B* (r=0.89; p=0.04) and *RORC* (r=0.5; p=0.04) in BC tissue. A similar tendency was also observed of *Foxp3* and *IL10* gene expressions. The assumption that the local inflammatory condition initiates miR-21 ex-

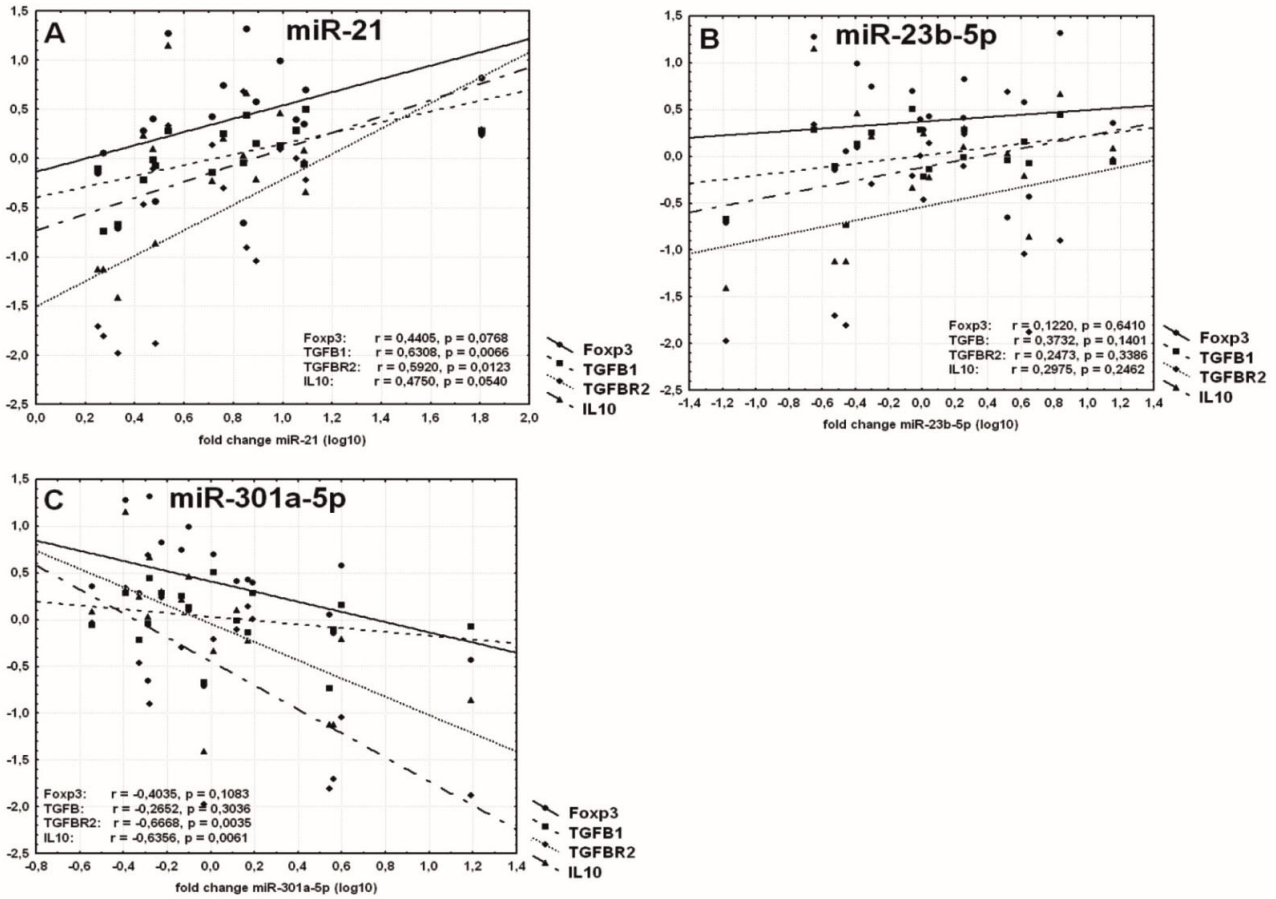

Figure 2. The correlation between miRs and Treg related genes. The correlation between miR-21 **(A)**, miR-23b-5p **(B)** and miR-301a-5p **(C)** and Treg related genes- *FOXP3, TGFBR2, TGFB1* and *IL10* in breast cancer tumor tissue. The results are expressed as log-transformed relative expression calibrated to non-tumoral tissue after normalization to references genes.

pression and the TGF-β signaling pathway ensures miR-21 biogenesis, and is in agreement with the observed positive relation with Th17 and Treg genes. We may suggest that miR-21 is involved in disturbing the anti-tumoral immune response in early carcinogenesis as well as in potentiation of tumor progression.

Concerning miR-301a-5p and miR-23b-5p expression we observed a significant positive association with larger tumor size (p=0.009; p=0.014, respectively), and with lymph node-negative status for miR-23b-5p (p=0.025). Additionally, miR-23b-5p was upregulated in 45%, and miR-301a-5p in 35% of the investigated tumoral tissue samples. These data suggest that 5p-arms of miR-23b and miR-301a also might be involved in breast carcinogenesis.

A comprehensive study by Pellegrino et al [7] showed that miR-23b enhances cell-cell adhesion and regulates BC cell motility and invasion in MCF-7 and in MDA-MB-231 cell lines by direct targeting the cytoskeletal reorganization. Hannafon et al [29] has demonstrated that the knockout of miR-23b/27b by CRISPR/Cas9 system reduces tumor growth *in vivo* in MCF-7 cells. They utilized sgRNAs designed to target within the stem-loop sequence of miR-23b and this knockout approach suggests the complete elimination of expression of both arms of miR-23b. These data are in general agreement with the observed positive association between tumor size and higher miR-23b-5p level in BC tissue in our study. On the other hand, miR-23b-5p was proved to be functional by direct binding to the proline oxidase mRNA 3'-untranslated region in renal cancer [21]. The proline metabolism axis was pointed out as a promising drug target against metastasis formation in BC [30]. MiR-23b-5p was previously explored in tissue and in serum exosomes in adenocarcinoma of the esophagus [21]. However, to the best of our knowledge, this is the first quantified study on miR-23b-5p in BC tissue. Based on the above, we assume that miR-23b-5p is involved in BC progression. In addition, the observed strong and significant positive correlation between miR-23b-5p and *IL12B* levels (r=0.9453;

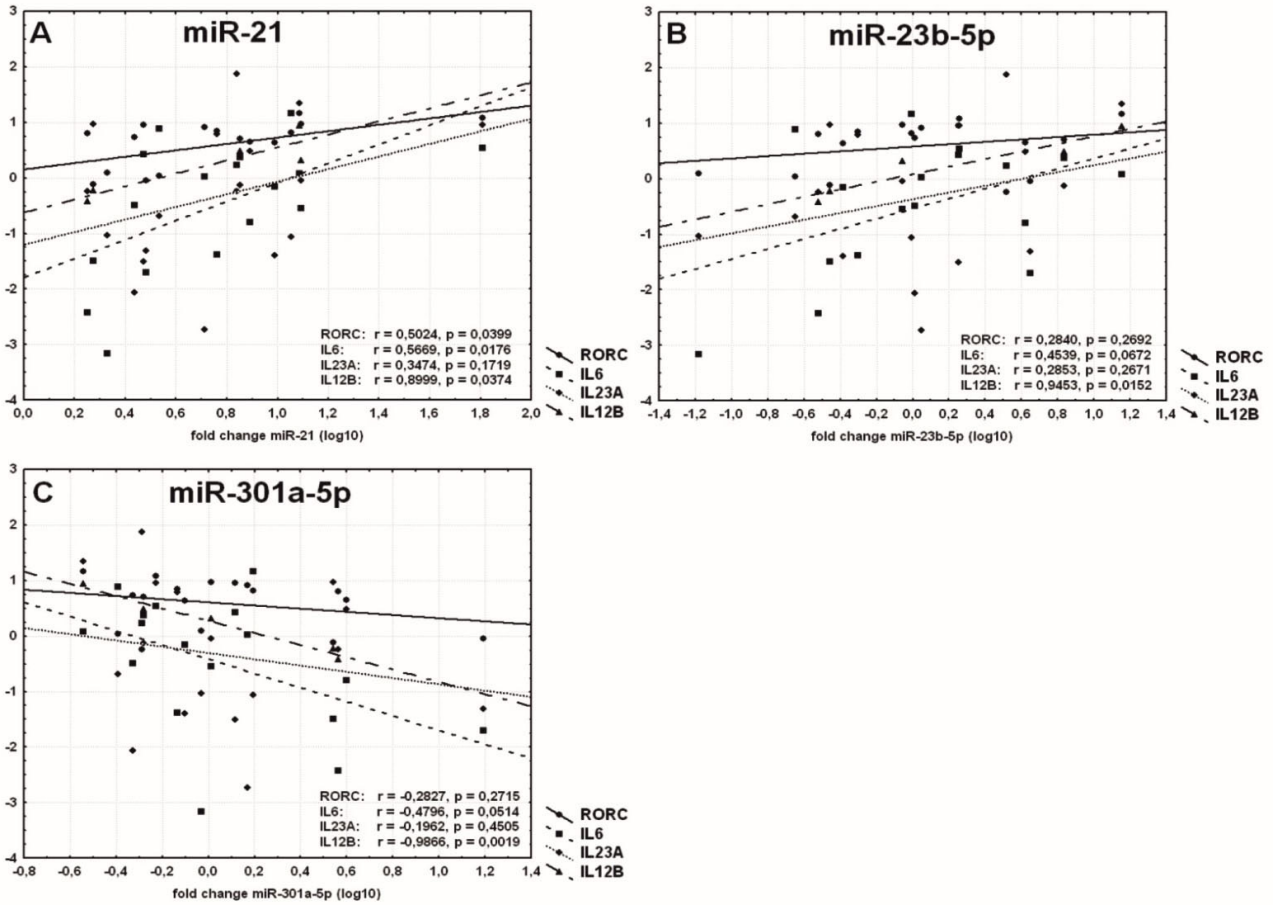

Figure 3. The correlation between miRs and Th17 related genes. The correlation between miR-21 **(A)**, miR-23b-5p **(B)**, and miR-301a-5p **(C)** and Th17 related genes- *RORC, IL23A, IL12B* and *IL6*. The results are expressed as log-transformed relative expression calibrated to non-tumoral tissue after normalization to references genes. The gene expression of *IL12B* was detected in 12 cases.

p=0.015) is in agreement with data provided by Pellegrino et al [7]. They have reported that primiR-23b is transcriptionally suppressed by AP-1. Our previous data [31] also demonstrated the augmented IL-12p40 production after inhibition of both JNK and p38 (as major mitogen-activated protein kinases regulate AP-1 activity) in peripheral blood mononuclear cells.

In contrast, miR-301a-5p was negatively correlated with *TGFBR2* (r=-0.6668; p=0.0035), *IL10* (r=-0.6356; p=0.0061) and *IL12B* (r=-0.9866; p=0.0019). In agreement, *TGFBR2* was among the predicted targets of miR-301a-5p by TargetScanHuman. Although the role of miR-301a-3p in the regulation of inflammatory response is widely explored [10], the involvement of this miR in the tumor-promoting or tumor-associated inflammation cannot be excluded. In a mouse model of colitis-associated cancer with the deleted gene of miR-301a (miR-301a–/– mice), Ma et al [11] demonstrated downregulated expression of IL-6, TNF-α and IL-17 during colon inflammation. Therefore, we may speculate that miR-301a-5p may facilitate proinflammatory response by suppressing the *TGFBR2* signaling. Furthermore, forkhead box L1 (FOXL1) transcription factor was confirmed as a direct target of miR-301a-5p. Recently, Wang et al [23] demonstrated that a long noncoding RNA, EPB41L4A-AS2, regulates FOXL1 through sponge-like binding with miR-301a-5p. Thus, the axis miR-301a-5p-FOXL1 affects the development and metastasis of hepatocellular carcinoma. The overexpression of FOXL1 was shown that inhibited the proliferation, invasion, and migration of BC through deactivating the Wnt/β-catenin signaling pathway [32]. Collectively, these data suggest that miR-301a-5p plays a role in regulating local inflammation and may affect breast carcinogenesis. In a view of the plasticity between different T cell lineages, including Th17-Treg, we suggest that miR-21 could be associated with Foxp3+ Treg transdifferentiation into Th17-like Treg cells and retained immunosuppressive phenotype, while miR-301a-5p might have an opposite effect.

The findings of our study, however, are subject to some limitations. First, we were focused on early-stages, luminal BC, and the observed associations might be different in HER2 overexpressing, triple-negative or metastatic BC. Second, the observed relation between miRNAs and target mRNAs levels should be confirmed by further functional analysis for verification of target genes. Although the correlativity did not explain the causality, it clearly shows the relation between mIRNAs and mRNA levels in the BC tissue and guides further research into clarifying the cause and the effect.

In conclusion, our study confirms the increased miR-21 level in tissues of early-stage, luminal BC and showed the role of miR-21 in breast cancer via affecting Th17-like Treg cancer promoting immunity. In addition, the obtained data suggested that 5p-arms of miR-23b and miR-301a also might be functional miRNAs having a role in tumor growth mediated by local Th17/ Treg imbalanced gene expression in BC.

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Conflict of interests

The authors declare no conflict of interests.

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