MicroRNA-10b and minichromosome maintenance complex component 5 gene as prognostic biomarkers in breast cancer

Sanaa Eissa · Marwa Matboli · Hanan H. Shehata ·
Nada O. E. Essawy

Abstract The aim of this study is to identify micro-ribonucleic acid (microRNA) and its target, in addition to their relationship to the outcome in breast cancer (BC). To achieve this aim, we investigated microRNA-10b (miR-10b) and minichromosome maintenance complex component 5 (*MCM5* mRNA) expression in 230 breast tissue samples by real-time PCR and semiquantitative conventional RT-PCR, respectively. Relapse-free survival (RFS) associated with miRNA-10b and *MCM5* mRNA were tested by Kaplan–Meier survival analysis. The impact of miRNA-10b and *MCM5* mRNA expression on the survival was evaluated by Cox proportional hazard regression model. The expression of miRNA-10b and *MCM5* mRNA was positive in 86.4 and 79.7 % breast cancer patients, respectively. The overall concordance rate between miRNA-10b and *MCM5* RNA was 90.4 %. The median follow-up period was 50 months. The survival analysis showed that high levels of both miR-10b and *MCM5* were associated with short relapse free survival of BC. We identified *MCM5* mRNA expression changes consistent with the miRNA-10b target regulation. Thus, we could consider miRNA-10b and *MCM5* mRNA as prognostic markers and potential therapeutic targets in breast cancer to be applied to other patient data sets.

S. Eissa (✉) · M. Matboli · H. H. Shehata
Oncology Diagnostic Unit, Medical Biochemistry and Molecular biology Department, Faculty of Medicine, Ain Shams University, Abbassia, P.O. Box 11381, Cairo, Egypt
e-mail: sanaaeissa@hotmail.com

N. O. E. Essawy
MSc. Pharmacogenetics and Stratified Medicine, University College London, London, UK

Abbreviations

miRNA	Micro-ribonucleic acids
MCM5	Minichromosome maintenance complex component 5
BC	Breast cancer
BMI	Body mass index
LN	Lymph node
ER	Estrogen receptor
PR	Progesterone receptor
Her-2 neu	Human epidermal growth factor receptor 2
IDC	Invasive duct carcinoma
ILC	Invasive lobular carcinoma
OCT	Oral contraceptive therapy
HT	Hormonal therapy

Introduction

Proliferation seems to be one of the most remarkable prognostic factors in breast cancer (BC), outlining the prognostic power of different genetic prognostic signatures and highlighting the clinical efficacy of proliferation-characteristic genes for BC prognosis, e.g., cyclin B1, Ki67, Myb-related protein B, surviving, and serine/threonine-protein kinase 6 [1]. Prognostic messenger RNA (mRNA) and miRNA expression signatures have been identified for specific breast tumor subtypes, but given the heterogeneity of patients' outcomes within the same subtype, pathways regulating tumor aggressiveness remain to be further clarified [2]. miRNA-10b is upregulated in metastatic breast cancer cells and positively control cell migration and invasion [3]. *MCM5* was identified as a member

of mini-chromosome maintenance family (MCM5) involved in DNA replication and cell proliferation [4].

Bioinformatics analyses indicate that a single miRNA can regulate several genes, highlighting the potential effect of miRNAs on different cellular pathways [5]. miRNAs have been shown to regulate several biological processes such as proliferation, development, apoptosis, and metastasis [6]. Oncogenic and tumor suppressive miRNAs have been involved in the control of critical cellular pathways. However, the identification of miRNA target activation/repression in tumor samples remains challenging [7].

We used bioinformatics tools in order to retrieve miRNA-10b and MCM5 mRNA expression, which have been recognized as a positively correlated set of miRNA-target pair. Such in silico data are based on previous microarray studies that integrated both the previous information gained from miRNA profiling and the microarray gene expression profiling of protein-coding genes (StarBase, http://starbase.sysu.edu.cn/) [3, 4]. Afterwards, we sought to confirm their differential expression in high- and low-grade tumors using quantitative real-time PCR (qRT-PCR) and conventional RT-PCR in 230 clinical breast tissue samples. Lastly, we investigated whether the expression of miRNA-10b and MCM5 mRNA could predict the clinical outcome of BC patients, thus resulting in the identification of new prognostic markers.

Patients and methods

Patients and clinical samples

This study was approved by the institutional review board of the Faculty of Medicine ethical committee, Ain Shams University, Cairo, Egypt. In addition, a written informed consent was provided by each patient approving the donation of her tissues left over after the performed diagnostic or therapeutic procedures.

All the patients were selected from the General Surgery Department, Ain Shams University Hospitals, in the period from January 2008 to August 2010. The patients were followed up for recurrence till September 2014.

This study investigated breast tissue samples from 118 breast cancer patients, 54 benign breast lesions (28 fibroadenoma, 11 duct ectasia, and 15 fat necrosis), and 58 healthy volunteers. All breast cancer patients undergoing primary breast tumor surgery were eligible for this study. Patients who had received chemotherapy or radiotherapy, as well as those who had inflammatory mastitis or other types of cancer, were excluded from the current study. Table 1 provides a summary of the clinical and biological characteristics of the study.

The follow-up was maintained through reviewing the clinical charts as well as getting in contact with the patients.

Local–regional relapses and the subsequent surgery occurring in the 90-day post-surgery period were considered part of the primary management. Relapses after 90 days were considered events, dated and reviewed by medical oncologists. Relapse is confirmed by detailed history of the primary tumor, its biology, management, and status at last follow-up; history of recurrent/metastatic disease including duration, previous sites of involvement, previous treatments, and their effect; histologically confirmed invasive breast cancer; ipsilateral axillary or internal mammary lymph node region recurrence after primary treatment with mastectomy or lumpectomy/quadrantectomy with clear surgical margins; a tumor recurrence in any soft tissue of the ipsilateral conserved breast or the chest wall; and mastectomy scar, and/or skin. Relapse does not include supraclavicular lymph nodes or tumor in the opposite breast.

All patients provided breast tissue samples. After surgical removal, all tissue samples were, immediately, flash-frozen in liquid nitrogen and stored at −70 °C until used. In addition, we analyzed all samples separately by histopathologists in order to grade and sub-classify the tumors based on TNM and World Health Organization classification [8–10]. Normal tissues were obtained from patients who underwent breast reduction surgery and confirmed using the classical pathology approaches.

Total RNA including miRNA extraction from breast tissue samples

Total RNA, including small noncoding miRNA, was isolated from breast tissue samples using miRNEasy RNA isolation kit (Qiagen, MD). Then, the RNA quality was determined using Ultraspec 1000, UV/visible spectrophotometer (Amersham Pharmacia Biotech, Cambridge, England) and NanoDrop 2000 (Thermoscientific).The ratio of absorbance at 260 and 280 nm was used to assess the purity of RNA. A ratio of ~2.0 is generally accepted as "pure" for RNA. Afterwards, the RNA was kept in −70 °C till its use in the reverse transcription polymerase chain reaction.

qRT-PCR analysis of miRNA-10b expression

One microgram miRNA was used in reverse transcription with a miScript II RT Kit (Qiagen/SABiosciences Corporation, Frederick, MD, USA). Quantitative RT-PCR was carried out using StepOnePlus™ System (Applied Biosystems Inc., Foster, CA, USA). Two small RNAs were used as the internal controls (RNU-6 and SNORD-68). The PCR primers for RNU-6, SNORD-68, and miR-10-b were purchased from (Qiagen, MD). SYBR Green Master Mix (Qiagen/SABiosciences Corporation, Frederick, MD, USA)was used in the real-time PCR reaction according to the manufacturer's suggested protocol; along with the manufacturer-provided miScript Universal primer and miRNA-specific forward

primer. The miRNA-specific primer (catalogue no. MS00031269,Qiagen) was chosen based on the miRNA sequences obtained from the miRbase database (http://microrna.sanger.ac.uk/) (details in Supplementary material).

Data normalization and quantification of microRNA in breast tissue samples

The expression level of microRNA was measured using the comparative Ct method [11] (details in Supplementary material).

Functional analyses of miRNA-10b

We chose to focus on miRNA10-b because, in the pathway enrichment analysis, it has got higher number of target genes, which are related to breast cancer than other upregulated miRNAs, StarBase (http://starbase.sysu.edu.cn/) and DIANA-mirPath software (Table 2) [12]. Although miRNA-10b has many putative target genes related to breast cancer, many are more related to other cancer types and others were extensively studied in literatures. We selected MCM5 mRNA

Table 1 Study population demographic and clinicopathological characteristics in the study (N=230)

Clinicopathological factors	Group			$\chi^2(p)$
	Malignant, no. (%)	Benign, no. (%)	Normal, no.(%)	
Age, mean (mean±SD)	52.58±13.3	49.78±13.2	50.5±11.16	F: 0.537[a], p: (0.586)
Parity				
Mutipara	98 (83.1 %)	46 (85.2 %)	53 (91.4 %)	2.207[b], p: (0.32)
Nullipara	20 (16.9 %)	8 (14.8 %)	5 (8.6 %)	
Menopausal				
Premenopausal	46 (39 %)	22 (40.7 %)	28 (48.3 %)	1.4[b], p: (0.494)
Postmenopausal	72 (61 %)	32 (59.3 %)	30 (51.7 %)	
Family history				
Positive	42 (35.6 %)	2 (3.7 %)	0 (0 %)	42.7[b], p: (0.001)[c]
Negative	76 (64.4 %)	52 (96.3 %)	58 (100 %)	
BMI				
Normal	30 (25.4 %)	34 (63 %)	36 (62.1 %)	52.75[b], p: (0.001)[c]
Overweight	36 (30.5 %)	16 (29.6 %)	20 (34.5 %)	
Obese	52 (44.1 %)	4 (7.4 %)	2 (3.4 %)	
OCT				
Past administration	67 (56.8 %)	22 (40.7 %)	25 (43.1 %)	5.108[b], p: (0.07)
Never	51 (43.2 %)	32 (59.3 %)	33 (56.9 %)	
HT				
Past administration	70 (59.3 %)	24 (44.4 %)	25 (43.1 %)	5.6[b], p: (0.06)
Never	48 (40.7 %)	30 (55.6 %)	33 (56.9 %)	
Molecular subtype				
Luminal A	52 (44.1 %)	–	–	
Luminal B	22 (18.6 %)			
Basal (triple negative)	30 (25.4 %)			
Her-2 overexpressing	14 (11.9 %)			
Histological type				
IDC	84 (72.2 %)	–	–	
ILC	2 (1.7 %)			
Mixed (IDC+ILC)	18 (15.3 %)			
Other	14 (11.9 %)			
Stage				
I	50 (42.3 %)	–	–	
II	56 (47.5 %)			
III	12 (10.2 %)			
Grade				
1	24 (20.4 %)	–	–	
2	74 (62.7 %)			
3	20 (16.9 %)			

BMI body mass index, OCT oral contraceptive therapy, HT hormonal therapy, IDC invasive duct carcinoma, ILC invasive lobular carcinoma

[a] One-way ANOVA test

[b] Chi-square test

[c] Significant correlation was detected between investigated groups at p<0.05 using chi-square test

Table 2 Pathway enrichment analysis of miRNA10b

KEGG pathway	Gene name	Found genes
Wnt signaling pathway	CTNNBIP1, BTRC, MAP3K7, NFAT5, CAMK2B	5
ErbB signaling pathway	PIK3CA, CRK, PAK7, CAMK2B	4
Cell adhesion molecules (CAMs)	ITGB8, SDC1, NFASC, CADM1	4
Focal adhesion	PIK3CA, ITGB8, FLT1, CRK, PAK7	5
MAPK signaling pathway	ATF2, BDNF, CRK, MAP3K7	5
Breast cancer	FAS,HLADP1,RASSF1,CAMK2B,PIK3CA,TGFBR1, PTEN,CYB11B2,AKAP10, MCM5	9

as a novel target directly related to replication and breast cancer (details in Supplementary material).

Semiquantitative conventional RT-PCR analysis of *MCM5* mRNA expression

RT-PCR reactions for *MCM5* were optimized in breast tissue samples using Qiagen One-Step RT-PCR Kit (Qiagen, USA). First-strand complementary DNA (cDNA) was synthesized from 2 μg total RNA using One-Step RT-PCR Kit (Qiagen, MD). *MCM5* primers (accession NM_006739.3; sense primer, 5′-CCCATTGGGGTATACACGTC-3′; antisense primer, 5′- CACGGTCATCTTCTCGCATCT -′3) were retrieved from e.PCR at Pubmed (available at: http://www.ncbi.nlm.nih.gov/gene/4174). In order to ensure the successful synthesis of complete cDNA strand, the latter was amplified for β-actin using primer pair (accession NM_ 001017992.2; sense, 5′-CTA CGT CGC CCT GGA CTT CGA GC -3′, and antisense, 5′-GAT GGA GCC GCC GAT CCA CAC GG -3′) [13]. The signal intensities observed in the agarose gel of *MCM5* RNA of each sample was determined relative to that of β-actin in the same sample (using "Quantity one" computer program version 4.6.3, Bio-Rad Laboratories, USA), thus determining the relative amount of *MCM5* RNA present in each sample [14] (details in Supplementary material).

Statistical analysis

Data analysis was performed using Statistical Package for the Social Sciences software (SPSS, Version 19, Chicago, IL, USA). Comparisons were performed using chi-squared and *t* tests or ANOVA tests, as appropriate (details in Supplementary material).

Results

Clinical utility of miRNA-10b expression in BC

The clinical characteristics of the participants and the pathology of cancers are listed in Table 1 with significantly statistical

Table 3 Differential expression of miRNA-10b, *MCM5*mRNA among malignant, benign, and control group in the study

Parameter	Group			
	Malignant (*N*=118)	Benign (*N*=54)	Normal (*N*=58)	Statistics
miRNA-10b				
Mean±SD	6.08±6.08	0.79±0.72	0.46±0.49	130.46[a], *p*: <.0001[b]
Mean rank	163.6	76.6	53.7	
Median	3.3	0.38	0.05	
No. of cases>1.52	102 (86.4 %)	8 (14.8 %)	0 (0 %)	147.26[c], *p*: <.0001[b]
MCM5mRNA				
Mean±SD	1.49±0.85	.29±0.40	0.20±0.33	103.75[a], *p*: <.0001[b]
Mean rank	157.9	76.8	74.09	
Median	1.6	0.002	0.002	
No. of cases>0.93	94 (79.7 %)	6 (11.1 %)	0 (0 %)	130.49[c], *p*: <.0001[b]

MCM5 minichromosome maintenance complex component 5

[a] Kruskal–Wallis test based on relative quantity (RQ)values of miRNA-10b and semiquantitive RT-PCR of MCM5

[b] Statistical significance was detected between the three studied groups (control, benign, and malignant) regarding miRNA-10b and MCM5 expression at *p*<0.0001
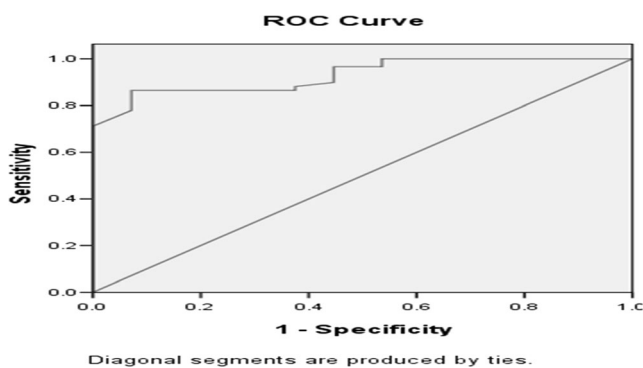
[c] Chi-square test

difference in the family history and body mass index among the groups of the study. We performed RT-qPCR using Syber green-based method in order to assess the breast tissue differential expression of hsa-miR-10b, thus validating its clinical utility for breast cancer prognosis. As summarized in Table 3, the median relative quantity (RQ) of miRNA-10b in the breast tissue samples of the validation groups were 0.05, 0.38, and 3.3 in healthy donors, benign, and malignant group, respectively. The detected miR-10b level was significantly lower in healthy donors and benign groups compared to breast cancer patients (Table 3).
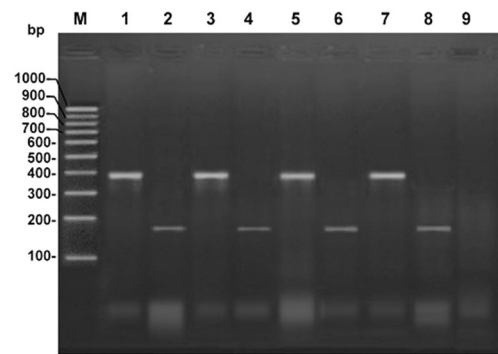
Receiver operating characteristic (ROC) curve analysis, based on RQ values, was performed to assess the sensitivity and the specificity of the hsa-miR-10b RT-qPCR assay in distinguishing breast cancer patients from the nonmalignant group. The obtained 95 % CI was (0.885–0.975), and the area under curve (AUC) was equal to 0.93. When the cutoff value was set to the optimal point, 1.52, the obtained specificity was 92.8 %, the sensitivity was 86.4 %, and the positive predictive value was 92.7 % (Table 3, Fig. 1). We did not find any significant correlation between the breast tissue miR-10b level and the different clinicopathological factors ($p>0.05$) (Supplementary Table 1).

Semiquantitaive conventional reverse transcriptase PCR for MCM5 gene expression

In this study, we investigated the miRNA and its target gene expression patterns in BC in order to identify candidate biomarkers for BC progression. Using semiquantitative RT-PCR, the mean rank levels for MCM5 RNA in benign and malignant groups were significantly increased by 1.02- and 2.13-fold as compared to the normal group, respectively ($p<0.0001$). The best cutoff point selected, using the ROC curve, for MCM5

Fig. 2 Semi-quantitative RT-PCR of the breast tissue MCM5 RNA and beta actin using agarose gel electrophoresis that produced bands with 172 and 385 bp, respectively. *Lane MW* molecular weight ladder standard (100 bp), *lanes 1–4* breast cancer tissue samples, *lanes 5–6* benign breast lesions, *lanes 7–8* normal breast tissue samples, *lane 9* negative control

RNA in order to discriminate between the malignant and the nonmalignant groups, was 0.9 (Figs. 2 and 3). Based on the mentioned cutoff value, 94 out of 118 (79.7 %) malignant patients were positive (> cutoff value), 6 out of 54 (11.1 %) benign patients were positive, while none of the healthy individuals were found to be positive (0 %) ($p<0.0001$) (Table 3). No significant correlation was found between MCM5 mRNA positivity rate and any of the studied clinicopathological factors ($p>0.05$) (Supplementary Table 1).

Concordance and correlation between miRNA-10b and its MCM5 mRNA target

The overall concordance rate between the miRNA-10b and MCM5 RNA was 90.4 % (Supplementary Table 2s). In addition, there was a significant positive correlation between the miRNA-10b and MCM5 mRNA ($p<0.001$, $r=0.236$) (Supplementary Table 3s).

Fig. 1 ROC curve analysis for *miRNA-10b* to calculate the best cutoff point that discriminates between malignant and nonmalignant groups. Best cutoff point of *miRNA-10b* was 1.52 [sensitivity=86.4 % , specificity=92.8 %, area under the curve (AUC) [SE]=0.3 [.023], 95 % confidence limits range=0.885–0.975, *P*<0.0001]
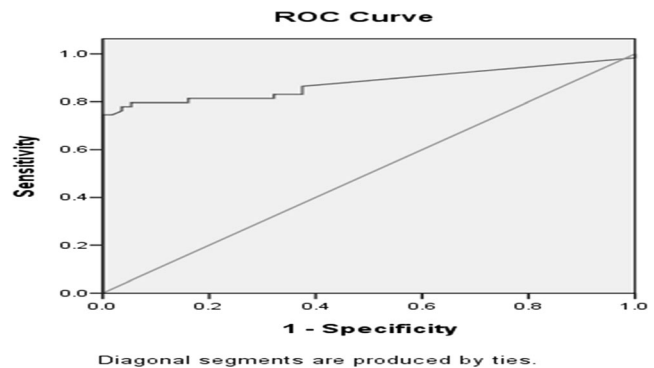

Fig. 3 ROC curve analysis for MCM5 mRNA to calculate the best cutoff point that discriminates between malignant and nonmalignant groups. Best cutoff point of MCM5 mRNA was 0.9 [sensitivity=79.7 % and specificity=94.6 %, area under the curve (AUC) [SE]=0.879 [.035], 95 % confidence limits range=0.809–0.948, *P*<0.0001]

Overall performance characteristics of all breast tissue investigated markers

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of miRNA-10b and *MCM5* mRNA as markers for discriminating between breast cancer and nonmalignant breast lesions were estimated as shown in Table 4. When miRNA-10b was tested independently using qRT-PCR, it showed the highest sensitivity (86.4 %) and specificity (92.7 %) in both the early stage and the low-grade tumors (Table 4). Moreover, the sensitivity of miRNA-10b/ *MCM5* mRNA pair increased to 89.8 %.

miRNA-10b and *MCM5* in relation to RFS of BC patients

In the univariate analysis, breast cancer patients with negative miRNA-10b and/or *MCM5* mRNA had relatively longer, statistically significant, RFS than patients with positive markers (Table 5). Kaplan and Meier analysis revealed significant decrease in RFS and increase in the cumulative hazards among miRNA-10b and *MCM5* mRNA-positive breast cancer patients (log rank test: chi square=12.4, 10.196; p=0.001, 0.001, respectively) (Supplementary Figure 2a, b and 3a, b). The results of Cox multivariate analysis showed that miRNA-10b and *MCM5* mRNA were independent prognostic factors of RFS (Supplementary Table 4s).

Discussion

Oncologists encounter exceptionally challenging task when it comes to taking clinical decisions on BC treatment. Such challenge could be tapered off if there are robust predictive

and prognostic factors, which guide the choice of treatment modalities [15]. Recently, molecular techniques, especially gene and miRNA expression profiling, have been used increasingly to improve BC classification and to evaluate patient prognosis and response to therapy[16].

In the current study, the bioinformatics prediction of miRNA-10b targets has revealed that it is linked to many genes involved in tumor proliferation, but many of them are

Table 5 Correlation of investigated prognostic markers and clinical outcomes of breast cancer patients

	Number	RFS (mean±SD) (months)	p value
ER			
Positive	72	48.7±6	0.58
Negative	46	49.4±7.2	
PR			
Positive	74	48.5±6.12	.25
Negative	44	49.8±7	
Her -2 neu			
Positive	34	47.5±6.8	.103
Negative	84	49.5±6.2	
MiRNA-10b			
Positive	102	47.9±6	.001**
Negative	8	55.5±5.45	
MCM5 mRNA			
Positive	94	48.2±6.5	.01*
Negative	24	51.9±5.45	
MiRNA-10b+*MCM5 mRNA*			
Positive	106	48.4±6.3	0.004*
Negative	6	54±5.4	

ER estrogen receptor, *PR* progesterone receptor, *Her-2 neu* human epidermal growth factor receptor, *MCM5* minichromosome maintenance complex component 5

*p<0.05, significant correlation; **p<0.01, highly significant correlation was detected between investigated groups using independent t test

Table 4 Performance characteristics of the investigated parameters in the breast cancer patients compared to benign and control groups of the study

Biomarker	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
Among investigated groups in the study(n=115)					
MiRNA-10b	86.4	92.8	92.7	86.7	89.6
MCM5 mRNA	79.7	94.6	94	81.5	87
MiRNA-10b+ MCM5 mRNA	89.8	91	91.3	89.4	90.4
Early stage (stage 0+1)(n=50)					
MiRNA-10b	92	92.8	85	96.3	92.5
MCM5 mRNA	80	94.6	87	91.3	90.1
MiRNA-10b+ MCM5mRNA	96	91	82.7	98	92.6
Low grade (grade 1) (n=24)					
MiRNA-10b	100	92.8	75	100	94.1
MCM5 mRNA	83.3	94.6	76.9	96.3	92.6
MiRNA-10b+ MCM5mRNA	100	91	70.6	100	92.6

MCM5 minichromosome maintenance complex component 5, *PPV* positive predictive value, *NPV* negative predictive value

related to other pathways than replication; some are more related to other cancer types, and others lacked novelty. Therefore, it drew our attention to explore miRNA-10b role in transcriptional activation on key oncogenes such as *MCM5*, a gene responsible for accelerating cell proliferation. For the first time, we presented integrated analysis combining miRNA-10b and *MCM5* mRNA expression. Our results show that miRNA expression sheds light on additional biological information beyond mRNA expression. In addition, we examined the extent of statistical correlation between *MCM5* mRNA and miRNA-10b expression in breast tissue samples. It is worth noting that the identification of such statistical correlation where BC cells can be targeted with miRNA, to turn on or off specific target genes, may have significant therapeutic potential in BC.

Although miRNA-10b was previously found to be down-regulated in a group of breast cancer samples [17], the upregulation of miR-10b in noninvasive breast cancer cells promoted epithelial mesenchymal transition and metastatic growth of these cells under tissue culture and in vivo conditions [18, 19]. Furthermore, therapeutic inhibition of miRNA-10b blocked metastatic growth in mouse model [20]. The fact that high expression levels of miR-10b were associated with a higher risk of recurrence concurs with recent findings reported by Chen and his colleagues [21], who documented miRNA-10b as a promising biomarker in detecting lymph node status of breast cancer.

Replication of mammalian genomes is a crucial decision point in human cell proliferation, which is strictly regulated by an intricate network of extracellular and intracellular signaling pathways [22]. Recent studies in *Xenopus* and yeast have identified origin recognition complex (ORC), cell division cycle 6 (Cdc6), and MCM proteins as essential factors for initiation of DNA replication in eukaryotic cells. Their role in growth regulation of human tissues and their respective tumors remains to be elucidated [23, 24]. In previous reports, *MCM5* mRNA was identified in cervical cancer [25] and colorectal cancer [26].

miRNA-10b seems to activate the transcription of *MCM5* gene by forming double-stranded RNA that match the promotor of the *MCM5* gene removing any inhibitory factors [27]. In the current study, miRNA-10b expression was statistically significantly associated with RFS and showed similar results in Kaplan–Meier analysis, for which patients ($n = 118$) were categorized on the basis of miR-10b RQ value. Moreover, Kaplan–Meier survival analysis revealed that *MCM5* expression had prognostic significance for both RFS and hazard ratio of recurrences. The prognostic significance was validated in multivariate analysis, too. Further large-scale prospective studies are required to validate their potential applicability for breast tumor prognosis, treatment, and surveillance.

Conclusion

The results obtained from this study may improve our understanding of the role of miRNA-10b and its selected target *MCM5* mRNA in relation to breast cancer invasiveness and ultimately lead to the identification of novel biomarkers associated with BC prognosis.

Conflicts of interest None

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