Clin. Lab. 2018;64:XXX-XXX

ORIGINAL ARTICLE

Analysis of miRNA-221 Expression Level in Tumors and Marginal Biopsies from Patients with Breast Cancer (Cross-Sectional Observational Study)

Atefe Abak [1, 2], Sima Amini [1, 2], Mehrdad A. Estiar [3], Vahid Montazeri [4], Ebrahim Sakhinia [1, 5, 6], Alireza Abhari [2, 7]

[1] *Division of Medical Genetics, Department of Biochemistry and Clinical Laboratory, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran*
[2] *Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran*
[3] *Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran*
[4] *Department of Thorax Surgery, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran*
[5] *Tuberculosis and Lung Disease Research Center, Tabriz University of Medical Sciences, Tabriz, Iran*
[6] *Tabriz Genetic Analysis Center (TGAC), Tabriz University of Medical Sciences, Tabriz, Iran*
[7] *Division of Clinical Biochemistry, Department of Biochemistry and Clinical Laboratory, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran*

SUMMARY

Background: **miRNA-221 and miRNA-222 are two homologous microRNAs, the high-expression levels of which have been commonly demonstrated in the most current human cancer types as well as breast cancer. The purpose of this research was to determine the clinical value of measuring the expression level of hsa-miR-221-3p in breast cancer tissues and evaluate its biological and prognostic importance in breast cancer (BC).**

Methods: **A total of 40 tumor samples and matched tumor-free margin specimens were obtained during surgery from patients with BC. After total RNA extraction and cDNA synthesis, the relative expression level of hsa-miR-221-3p in tumor and marginal tissues was examined by quantitative real-time PCR. Moreover, the association between hsa-miR-221-3p expression and clinicopathological features of patients was detected.**

Results: **The relative expression level of hsa-miR-221-3p in BC tissues was significantly higher than that in adjacent noncancerous breast biopsies ($p \leq 0.0001$). Also, there was no significant association between hsa-miR-221-3p expression with clinicopathological characteristics ($p > 0.05$). The receiver operating characteristic (ROC) curve analyses also represented an optimum cutoff point of < 4.34 to show that hsa-miR-221-3p is an effective molecular biomarker for BC diagnosis.**

Conclusions: **This study illustrated that analysis of hsa-miR-221-3p relative gene expression may be applied as a biomarker for screening BC patients and could be a substantial tool in diagnosis and prognosis. Also, that could be advantageous in decreasing surgical mistakes in tumor elimination through the surgery and enhancing all over the progression of surgery with reformed tumor clearance.**

(Clin. Lab. 2018;64:xx-xx. DOI: 10.7754/Clin.Lab.2017.170821)

Correspondence:
Ebrahim Sakhinia
Division of Medical Genetics
Department of Clinical Biochemistry
Faculty of Medicine
Tabriz University of Medical Sciences
Tabriz
Iran
Email: esakhinia@yahoo.co.uk

Alireza Abhari
Division of Clinical Biochemistry
Department of Biochemistry and Clinical Laboratory
Faculty of Medicine
Tabriz University of Medical Sciences
Tabriz
Iran
Email: abharial@tbzmed.ac.ir

Manuscript accepted September 26, 2017

KEY WORDS

hsa-miR-221-3p, microRNA, breast cancer, quantitative real-time PCR, biomarker

INTRODUCTION

Breast cancer (BC) is a major health problem for women around the world, including in Iran as each year more than 502,000 women, because of the risk of this cancer, will lose their lives [1]. BC is diagnosed by local invasion and primary metastasis, thus displaying low sensitivity to chemotherapy agents [2,3]. In spite of the advances of modern treatment based on chemotherapy and radiotherapy, a low rate of long-term survival and functional status of patients has remained in cases with progressed BCs [4]. Currently, molecular methods, especially gene expression profiling, indicated that the altered hormone receptor expression depended on constant "molecular portraits" in cancer tissues which are detected considerably in tumorigenesis and can display significant factors of cancer biology. They have been used to improve different BCs care and to determine patient prognosis and response to therapy [5,6].

MicroRNAs (miRNAs) are evolutionarily conserved, small (18 - 25 nt), single-stranded molecules that inhibit the expression of protein-coding genes by translational repression, messenger RNA degradation, or both [7]. The miRNAs mediate their regulatory function via imperfect binding to the 3´-UTR region of target mRNAs carrying the complementary sites [8]. Further miRNA discovery and a majority of evidence of their common regulatory functions in the cellular processes have presented additional insight into regulating gene expression [9]. Aberrant miRNA expression is relevant to the regulation of oncogenes or tumor suppressor genes implicated in the cell signaling processes relevant to several diseases, especially cancer. Although miRNAs regulate cancer cell proliferation, differentiation, metastasis and survival, controlling for their expression levels renders a perfect remedial strategy towards cancer development. Thus, miRNAs can be considerable candidates for new diagnostic markers and remedial interventions in cancer as recently represented in other miRNA controlled diseases [10]. miRNA-221 is the most over-expressed miRNA in human cancers in an extensive range including breast cancer [11], glioblastoma, bladder, pancreatic, hepatocellular, thyroid, gastric and prostate carcinomas [12]. In human DNA, chromosome Xp11.3 is the location of the miRNA-221 and miRNA-222 gene cluster in tandem, which is transported as far as 726 bp and has been identified to be over expressed in multiple kinds of cancer. Per to their seed sequence similarity, both miRNAs have been distinguished to directly target P27-kip1, Bmf, PTEN, Mdm2, PUMA, TRPS1, Fos and AKT3 [12-19]. MiRNA-221 and miRNA-222 in breast cancer have been indicated to contribute in control of ERα expression, inhibition of ERα-mediated signaling and drug resistance procedures [20-24].

The purpose of this study was to assay the relative expression level of has-miR-221-3p through a quantitative reverse transcription PCR (qRT-PCR) in BC tissue specimens, to determine its clinical importance and to estimate the potential competence of has-miR-221-3p as a prognostic biomarker.

MATERIALS AND METHODS

Study design

The present cross-sectional research was undertaken as a collaboration between the Biotechnology Research Center of Tabriz University of Medical Sciences and the Noor Nejat Hospital (Tabriz, Iran). The ethical protocol of the research was approved by the Ethical Committee of Tabriz University of Medical Sciences (TUMS), according to the Declaration of Helsinki. Written informed consent was obtained from all patients participating in this research.

Patients and clinical specimens

The participants in this research were Iranian female patients with the same ethnicity and geographical residency. Forty pairs of breast cancer specimens and adjacent normal tissues were collected during surgeries for quadrantectomy and mastectomy, from patients referred to Noor Nejat Hospital (Tabriz, Iran). The marginal biopsies were obtained from a segment of the limited sample at the farther interval from tumor (> 2 cm from the tumor). All breast cancer patients who initially underwent breast tumor surgery were qualified for this study. Inclusion criteria were women aged > 30 years and had unilateral breast cancer and all the patients mentioned in this study who had not received any chemotherapy agents or other types of therapy before the surgical operation. The exclusion criteria included prior chemotherapy, radiation therapy, immunotherapy and patients who had inflammatory mastitis or other types of human cancer disease. All the tissue specimens were obtained instantly after resection and placed immediately into RNAlater (Qiagen, Hilden, Germany) and then flash frozen in liquid nitrogen and stored at -80°C up to RNA extraction. Clinical and pathological information documented at the time of operation contained the age, BMI, tumor size, histological type, tumor grade, menopausal status, lymph node metastasis and BC stages. Tumors were staged according to the American Joint Committee on Cancer (AJCC) TNM staging [25]. The histological assortment was based on the WHO assortment system [26] and was validated by the pathologist.

MiRNA extraction from BC tissue specimens

MiRNA was extracted from breast tissue specimens with miRNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions to gain the miRNAs from 40 - 50 mg total RNA specimens. The quantity and concentration of the RNAs were measured

by a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). A ratio of absorbance at 260 nm and 280 nm ~ 2.0 is admitted as "pure" for RNA. Therefore, the extracted RNAs were stored in -70°C until utilization in the cDNA synthesis. The quality of the RNAs was scored using agarose gel electrophoresis stained with SYBR Safe dye (Invitrogen, USA).

Quantitative real-time PCR assay of hsa-miR-221-3p relative expression

For the reverse transcription, 2 µg miRNA was utilized with a miScript II RT Kit (Qiagen, Hilden, Germany). Real-time quantitative PCR (qRT-PCR) was performed using Rotor Gene (Corbett/Qiagen, Hilden, Germany). The HS_RNU6-6p was elected as the internal control. The PCR primers for HS_RNU6-6p and hsa-miR-221-3p were obtained from (Qiagen, Hilden, Germany). The expression of hsa-miR-221-3p was assessed by qRT-PCR analysis utilizing miScript SYBR Green PCR kit (Qiagen, Hilden, Germany); along with the manufacturer provided miScript universal primer and microRNA-specific forward primer according to the manufacturer's suggested protocol. The hsa-miR-221-3p specific primer (Qiagen, Hilden, Germany) was elected based on the microRNA sequences retrieved from the miRbase database (http://microrna.sanger.ac.uk/).

Data normalization and quantification of hsa-miR-221-3p in BC tissue specimens

Normalization using the housekeeping RNU6-2 gene was applied because of the expression level of the RNU6-2 gene. The expression levels of the housekeeping gene (RNU6-2) and the hsa-miR-221-3p genes were evaluated by real-time quantitative PCR (qRT-PCR). The expression level of hsa-miR-221-3p was determined according to the cycle threshold (Ct), and relative expression levels of hsa-miR-221-3p were defined by applying the $2^{-\triangle\triangle Ct}$ assays method after normalization to the expression of SNU6-2 [27]. The standard curves, which were plotted, represented the role of the procedure to precisely calculate the expression levels of the hsa-miR-221-3p gene.

Statistical analysis

The expression level of hsa-miR-221-3p in BC tissues compared to marginal noncancerous tissues was analyzed using the Pffafl formula. The statistical discrepancy in hsa-miR-221-3p expression in tumor and matched non-tumor counterparts was assayed via paired samples t-test. Also, Pearson's correlation test was applied for measuring the correlation between the expression level of has-miR-221-3p and patient clinical characteristics. Kolmogorov-Smirnov Z normality test for estimating normality of data and Levene's Test for accounting Equality of Variances were utilized. The association between hsa-miR-221-3p expression and clinicopathological parameters was evaluated by t-test between two groups. All tests were accomplished using SPSS version 23 statistical software (SPSS Inc., Chicago, IL, USA). In addition, a receiver operating characteristic (ROC) curve was created by applying Sigma plot v.12.5 (Systat Software Inc, San Jose, CA, USA) to measure the specificity and sensitivity of prognosticating breast cancer (BC) and normal tissue through hsa-miR-221-3p.

RESULTS

Expression level of hsa-miR-221-3p in BC tissue specimens

Quantitative real-time polymerase chain reaction assay of hsa-miR-221-3p was accomplished in 40 pairs of BC tissues and marginal noncancerous tissues. The results demonstrated that hsa-miR-221-3p expression levels were considerably increased in BC tissues compared with normal noncancerous matches (fold change = 3.19; $p \leq 0.0001$). Comparisson of hsa-miR-221-3p gene expression level differences is represented for tumor and marginal noncancerous tissues in Figure 1. The value of $\triangle$Ct (mean ± SD) for hsa-miR-221-3p was 5.19 ± 1.14 in BC tissues and 6.98 ± 1.57 in marginal noncancerous tissues.

Capability of has-miR-221-3p to play as a BC tumor biomarker role

Receiver Operating Characteristics (ROC) curves were created. To estimate the specificity and sensitivity of predicting BC and marginal tissue specimens the area under the curve (AUC) was calculated through hsa-miR-221-3p expression level (Figure 2). According to the ROC curve assays, hsa-miR-221-3p indicated a ROC area (AUC) of 0.81 (Figure 3; $p \leq 0001$). The plot demonstrated specificity and sensitivity at various cutoff points. Also, we accomplished a post-test from pre-test probability of 0.5 and cost ratio of 1.00 to calculate the optimal cutoff value. The optimal cutoff point was < 4.34 for hsa-miR-221-3p with 0.23 and 1.00 sensitivity and specificity, respectively.

Correlation between hsa-miR-221-3p expression level and clinicopathological characteristics of BC patients

Independent sample t-test illustrated no association between hsa-miR-221-3p expression level and clinicopathological characteristics of BC patients, including age, BMI, tumor size, histological type, menopausal status, lymph node metastasis, tumor grade, and BC stages according to the AJCC classification (Table 1).

DISCUSSION

Each miRNA has a different expression pattern in various breast cancer phenotypes. Thus, it can be utilized for diagnosis, prognosis, tumor classification and therapy [28]. Emerging evidence illustrated that the oncomiR, miRNA-221, is constantly highly-expressed in

Table 1. Relationships between miRNA-221 expression level in BC tissue samples and clinicopathological features of BC patients.

Manifestation	N	MicroRNA-221 (ΔCt)	p-value
Age			
< 50 years	25	5.32 ± 1.08	0.38
> 50 years	15	4.97 ± 1.26	
BMI			
Normal (18.5 to 24.9)	23	5.12 ± 1.21	0.49
Overweight (25 to 29.9)	17	5.28 ± 1.08	
Tumor Size			
< 2.5 cm	26	5.12 ± 1.15	0.96
> 2.5 cm	14	5.31 ± 1.16	
Histological Type			
IDC	35	5.15 ± 1.14	0.75
ILC	5	5.43 ± 1.27	
Tumor Grade			
I, II	34	5.18 ± 1.17	0.48
III	6	5.21 ± 1.09	
Tumor Stage			
I, II	24	5.45 ± 1.05	0.49
III, IV	16	4.79 ± 1.20	
Lymph Node Metastasis			
Positive	26	5.03 ± 1.10	0.83
Negative	14	5.48 ± 1.20	
Menopausal			
Premenopausal	28	5.42 ± 1.14	0.57
Postmenopausal	12	4.64 ± 0.99	

Ct - cycle threshold, BC - breast cancer, BMI - body mass index, IDC - invasive ductal carcinoma, ILC - invasive lobular carcinoma.

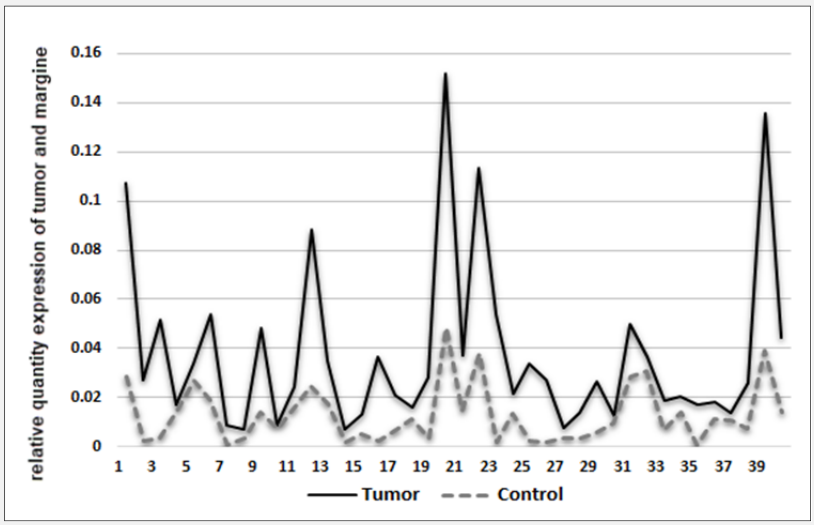

Figure 1. Comparison of the expression level of miRNA-221 in Breast cancer (BC) tissues and normal tissue margins.

The expression level of miRNA-221 was significantly increased in the BC tissues compared with normal tissue margins (fold change = 3.19; $p \leq 0.0001$). The x-axis represents the tumor and marginal non-tumor samples and the y-axis represents the relative expression of these samples compared with the expression of the internal control gene (RNU6-2). The upper line graph, which has sharp edges and higher values, is related to tumor tissues and the lower line graph indicates healthy marginal tissues.

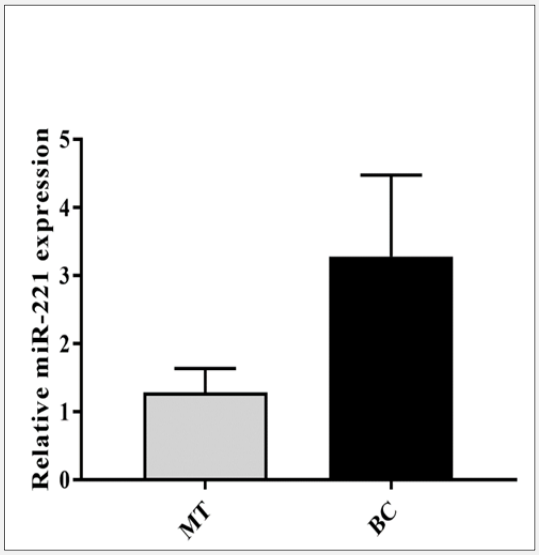

Figure 2. Relative expression level of miRNA-221 in BC tissues and normal tissue margins ($p \leq 0.0001$).

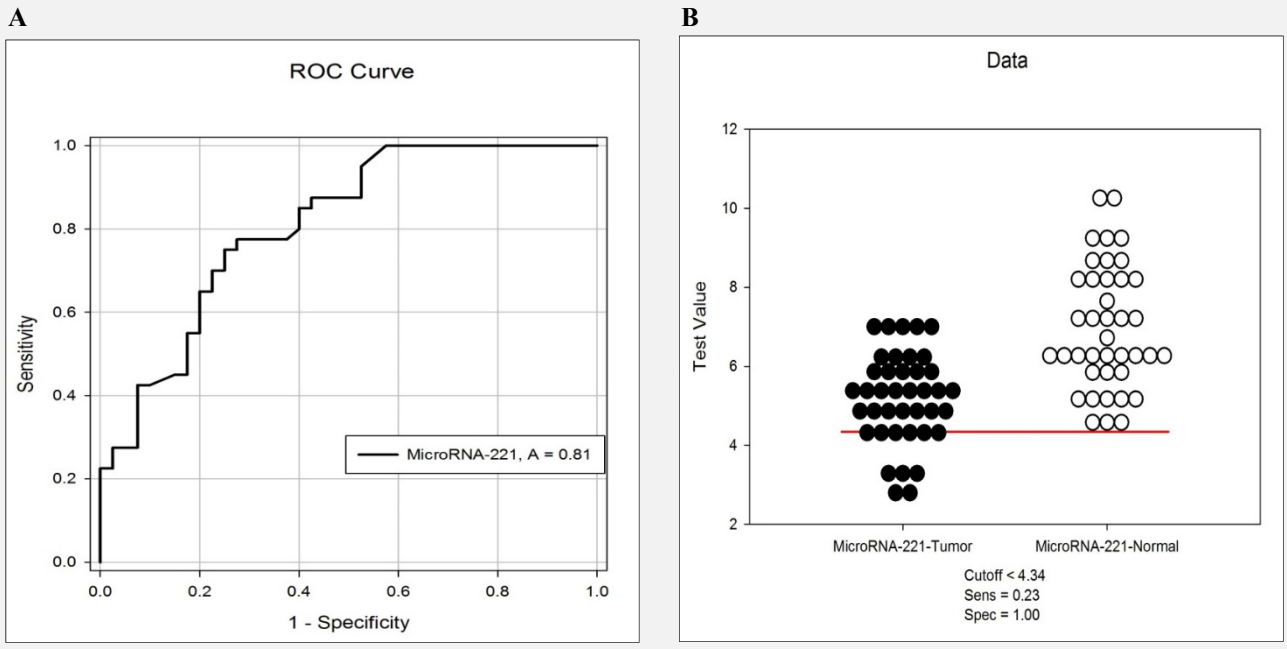

Figure 3. The receiver operating characteristic (ROC) curve was automatically generated from 36 points of cutoff values set by Sigma Plot software.

(A) The area under the curve (AUC) was 0.81 out of 1.00 for miRNA-221. (B) The dot histogram shows the optimum cutoff points for miRNA-221 in tumor and normal specimens.

solid and liquid tumor malignancies of various tissue origins. MiRNA-221 and its regulatory targeted genes have become the new insight of cancer remedy. The miRNA-221 is a potential oncogenic miRNA, which is placed on human chromosome X and highly-expressed in several invasive carcinomas e.g., of prostate cancer, pancreatic cancer, hepatocellular carcinoma and papillary thyroid carcinoma [29].

It has been illustrated that there is a high-expression of miRNA-221 effectively promoting tumor cell progression via containing cell lines to access the S phase of cell cycle [30]. The bioinformatics provision in the recent study disclosed that miRNA-221 targets are dependent on multiple genes due to carcinogenesis. Even more, through one of the indicating reports, miRNA-221 expression level was highly-expressed in breast cancer tissue specimens and was linked to advanced clinical stage of the breast tumor [11]. Furthermore, the current study showed that cells that tolerate EMT during tumorigenesis are mostly discrete from the primary tumor, invade through the basement membrane (BM) into blood circulation, and create metastasis at a secondary tumor size [31,32]. It is now obvious that miRNA-221 regulates EMT in cancer progression [33]. Interestingly, another research showed that miRNA-221, low-expressed in prostate cancer tissues, is based on the presence of the oncogenic transmembrane prostate serine 2: ETS-related gene (TMPRSS2: ERG) fusion transcript. miRNA-221 in prostate cancer tissues illustrates a different role distinct from other cancer types [34]. Also, miRNAs can be used as biomarkers for other human diseases including oxidative stress in polytrauma patients for the remedial management in the absence of a strict assessment of the biological changes [35]. In addition, various researchers have found that correlations exist between the microRNAs and the NF-κB activity in the case of polytrauma patients with sepsis [36].

The results of the recent study confirm the supposition that miRNA-221 is relevant to the etiology of breast cancer. This study proposed to assay expression of hsa-miR-221-3p in tumor specimens compared to non-tumor counterparts, and finally, to utilize this as a significant prognostic biomarker in early diagnosis for breast cancer patients. We performed quantitative real-time polymerase chain reaction for hsa-miR-221-3p transcripts on 40 tumor and marginal noncancerous tissues. Our results represented that hsa-miR-221-3p was remarkably up-regulated in breast cancer specimens compared with non-tumor counterpart tissues ($p \leq 0.0001$). Furthermore, we assayed the correlation between hsa-miR-221-3p expression level and clinicopathological features of BC patients. Although, there were no significant relationships between expression levels of hsa-miR-221-3p and clinicopathological features, including age, BMI, tumor size, histological type, menopausal status, lymph node metastasis, tumor grade and tumor stage. We also calculated the capability of hsa-miR-221-3p expression level to measure the specificity and sensitivity of predicting BC and normal tissues. The optimal cutoff point was < 4.34 for hsa-miR-221-3p with 0.23 and 1.00 as sensitivity and specificity, respectively.

CONCLUSION

In conclusion, from these results, our principle result is that hsa-miR-221-3p was highly-expressed in BC tissue specimens in comparing with marginal non-cancerous specimens and no meaningful relevance was acquired between the expression level of hsa-miR-221-3p and clinicopathological features of BC patients. The hsa-miR-221-3p specificity and sensitivity in optimal cut-off point confirmed that hsa-miR-221-3p is a helpful molecular biomarker in BC diagnosis. Thus, collectively, hsa-miR-221-3p expression level may display an effective biomarker for BC patient's prognosis. Further insights into the functional and clinical implications of hsa-miR-221-3p may contribute to early breast cancer detection and diagnosis helping to determine BC therapeutic approaches.

Acknowledgement:
This work was supported by funds from the Biotechnology Center of Tabriz University of Medical Sciences, Iran as an MSc thesis (The thesis number: 93/2-7/4). The authors are thankful to Professor of Surgery, Dr. Vahid Montazeri at Noor Nejat Hospital for providing the surgical breast tissue specimens and patients information.

Declaration of Interest:
The authors declared no conflicts of interest.

References:

1. Mousavi SM, Gouya MM, Ramazani R, Davanlou M, Hajsadeghi N, Seddighi Z. Cancer incidence and mortality in Iran. Ann Oncol. 2009;20(3):556-63 (PMID: 19073863).

2. Gradishar WJ. Treatment of metastatic breast cancer. J Natl Com pr Canc Netw. 2014;12(5S):759-61 (PMID: 24853211).

3. Martin HL, Smith L, Tomlinson DC. Multidrug-resistant breast cancer: current perspectives. Breast Cancer (Dove Med Press). 2014;6:1-13 (PMID: 24648765).

4. Sørlie T, Perou CM, Fan C, et al. Gene expression profiles do not consistently predict the clinical treatment response in locally advanced breast cancer. Mol Cancer Ther. 2006;5(11) 2914-18 (PM ID: 17121939).

5. Cava C, Bertoli G, Ripamonti M, et al. Integration of mRNA expression profile, copy number alterations, and microRNA expression levels in breast cancer to improve grade definition. PloS one 2014;9(5):e97681 (PMID: 24866763).

6. Ergun S, Tayeb TS, Arslan A, et al. The investigation of miR-221 -3p and PAK1 gene expressions in breast cancer cell lines. Gene 2015;555(2):377-81 (PMID: 25447917).

7. Pillai RS, Bhattacharyya SN, Filipowicz W. Repression of protein synthesis by miRNAs: how many mechanisms? Trends Cell Biol. 2007;17(3):118-26 (PMID: 17197185).

8. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009;136(2):215-33 (PMID: 19167326).

9. Kloosterman WP, Plasterk RH. The diverse functions of microRNAs in animal development and disease. Dev Cell. 2006;11(4): 441-50 (PMID: 17011485).

10. Garofalo M, Quintavalle C, Romano G, M Croce C, Condorelli G miR221/222 in cancer: their role in tumor progression and response to therapy. Curr Mol Med. 2012;12(1):27-33 (PMID: 220 82479).

11. Eissa S, Matboli M, Sharawy A, El-Sharkawi F. Prognostic and biological significance of microRNA-221 in breast cancer. Gene 2015;574(1):163-67 (PMID: 26253160).

12. Galardi S, Mercatelli N, Giorda E, et al. miR-221 and miR-222 expression affects the proliferation potential of human prostate carcinoma cell lines by targeting p27Kip1. J Biol Chem. 2007;28 2(32):23716-24 (PMID: 17569667).

13. Gramantieri L, Fornari F, Ferracin M, et al. MicroRNA-221 targets Bmf in hepatocellular carcinoma and correlates with tumor multifocality. Clin Can Res. 2009;15(16):5073-81 (PMID: 19671 867).

14. Garofalo M, Di Leva G, Romano G, et al. miR-221&222 regulate TRAIL resistance and enhance tumorigenicity through PTEN and TIMP3 downregulation. Cancer Cell. 2009;16(6):498-509 (PMID: 19962668).

15. Kim D, Song J, Jin E-J. MicroRNA-221 regulates chondrogenic differentiation through promoting proteosomal degradation of slug by targeting Mdm2. J Biol Chem. 2010; 285(35):26900-07 (PMID: 20576614).

16. Sun T, Wang Q, Balk S, Brown M, Lee G-SM, Kantoff P. The role of microRNA-221 and microRNA-222 in androgen-independent prostate cancer cell lines. Cancer Res. 2009;69(8):3356-63 (PMID: 19351832).

17. Zhang C, Zhang J, Zhang A, et al. PUMA is a novel target of miR -221/222 in human epithelial cancers. Int J Oncol. 2010;37(6): 1621 (PMID: 21042732).

18. Stinson S, Lackner MR, Adai AT, et al. TRPS1 targeting by miR-221/222 promotes the epithelial-to-mesenchymal transition in breast cancer. Sci. Signal. 2011;4(177):ra41-ra41 (PMID: 216733 16).

19. Errico MC, Felicetti F, Bottero L, et al. The abrogation of the HOXB7/PBX2 complex induces apoptosis in melanoma through the miR-221&222-c-FOS pathway. Int J Cancer. 2013;133(4):879 -92 (PMID: 23400877).

20. Miller TE, Ghoshal K, Ramaswamy B, et al. MicroRNA-221/222 confers tamoxifen resistance in breast cancer by targeting p27-Kip1. J Biol Chem. 2008;283(44):29897-903 (PMID:18708351).

21. Lu Y, Roy S, Nuovo G, et al. Anti-microRNA-222 (anti-miR-222) and-181B suppress growth of tamoxifen-resistant xenografts in mouse by targeting TIMP3 protein and modulating mitogenic signal. J Biol Chem. 2011;286(49):42292-302 (PMID: 220097 55).

22. Cochrane DR, Cittelly DM, Howe EN, et al. MicroRNAs link estrogen receptor alpha status and Dicer levels in breast cancer. Horm Cancer. 2010;1(6):306-19 (PMID: 21761362).

23. Network NCC. Breast cancer Clinical Practice Guidelines in Oncology. J Natl Compr Canc Netw. 2003 Apr;1(2):148-88. (PMID: 19768876).

24. Compton CC, Byrd DR, Garcia-Aguilar J, Kurtzman SH, Olawaiye A, Washington MK. Breast: Springer; 2012.

25. Livak KJ, Schmittgen TD. Analysis of relative gene expression d ata using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-08 (PMID: 11846609).

26. Paraskevopoulou MD, Georgakilas G, Kostoulas N, et al. DIANA -microT web server v5.0: service integration into miRNA functional analysis workflows. Nucleic Acids Res. 2013;41(W1):W16 9-W73 (PMID: 23680784).

27. Macfarlane LA, Murphy PR MicroRNA: Biogenesis, Function an d Role in Cancer. Curr Genomics. 2010 Nov;11(7):537-61. (PMID: 21532838).

28. Tanaka R, Tomosugi M, Horinaka M, Sowa Y, Sakai T. Metform in causes G1-phase arrest via down-regulation of MiR-221 and enhances TRAIL sensitivity through DR5 up-regulation in pancre atic cancer cells. PloS one 2015;10(5):e0125779 (PMID: 259558 43).

29. Mardente S, Mari E, Consorti F, et al. HMGB1 induces the over expression of miR-222 and miR-221 and increases growth and motility in papillary thyroid cancer cells. Onc Rep. 2012;28(6): 2285-89 (PMID: 23023232).

30. Pineau P, Volinia S, McJunkin K, et al. miR-221 overexpression contributes to liver tumorigenesis. Proc Natl Acad Sci U S A 2010;107(1):264-69 (PMID: 20018759).

31. Thiery JP, Sleeman JP. Complex networks orchestrate epithelial-mesenchymal transitions. Nat Rev Mol Cell Biol. 2006;7(2):131-42 (PMID: 16493418).

32. Lim J, Thiery JP. Epithelial-mesenchymal transitions: insights from development. Development 2012;139(19):3471-86 (PMID: 22949611).

33. Lambertini E, Lolli A, Vezzali F, Penolazzi L, Gambari R, Piva R Correlation between Slug transcription factor and miR-221 in MDA-MB-231 breast cancer cells. BMC Cancer. 2012;12(1):1 (PMID: 23031797).

34. Gordanpour A, Stanimirovic A, Nam RK, et al. miR-221 Is down -regulated in TMPRSS2: ERG fusion-positive prostate cancer. Anticancer Res. 2011;31(2):403-10 (PMID: 21378318).

35. Bedreag OH, Sandesc D, Chiriac SD, et al. The Use of Circulating miRNAs as Biomarkers for Oxidative Stress in Critically Ill Polytrauma Patients. Clin Lab. 2016;62(3):263-74 (PMID: 27156 313).

36. Papurica M, Rogobete AF, Sandesc D, et al. The Expression of Nuclear Transcription Factor Kappa B (NF-κB) in the Case of Critically Ill Polytrauma Patients with Sepsis and Its Interactions with microRNAs. Biochem Genet. 2016;54(4):337-47 (PMID: 27003424).