Kinetics of Circulating Levels of miR-195, miR-155 and miR-21 in Patients with Breast Cancer Undergoing Mastectomy

MARIA IGGLEZOU[1], KATERINA VARELI[1,2], GEORGIOS K. GEORGIOU[3], IOANNIS SAINIS[1] and EVANGELOS BRIASOULIS[1]

[1]Cancer Biobank Center and [2]Department of Biological Applications and Technologies, University of Ioannina, Ioannina, Greece;
[3]Department of Surgery, University Hospital of Ioannina, Ioannina, Greece

Abstract. *Background: MicroRNAs are small RNA molecules that negatively regulate the expression of the majority of proteins, mainly at the post-transcriptional level. Being stable in the circulation and resistant to storage handling, they are potentially promising biomarkers. Materials and Methods: We measured RNA levels of three microRNAs with tumorigenic or angiogenic potential (miR-155, miR-195, and miR-21) in blood samples taken from patients with early breast cancer, both preoperatively and postoperatively. Results: We found that persistently elevated postoperative levels of miR-195 were detected only in patients who developed early tumor relapse and that miR-155 levels tended to increase three days postoperatively ($p=0.05$) and fell below baseline one month post-surgery ($p<0.05$). We had no major findings for miR-21. Conclusion: The results of this pilot study indicate a possible involvement of miR-155 in surgery-induced angiogenesis and potential prognostic significance of high postoperative levels of circulating miR-195 in patients with breast cancer.*

Breast cancer ranks second among the leading cancers causing death in women (1). The estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) are validated immunohistochemical markers that currently guide breast cancer therapy (2, 3). However, the search for new molecular biomarkers that can help early detection and guide post-surgical management of breast cancer is of great importance (2, 4).

MicroRNAs are a class of small non-coding RNAs (20-24 nucleotides) that control the expression of multiple genes at the post-transcriptional level by affecting the stability of and suppressing the translation of mRNAs. It is noteworthy that a single miRNA may have more than 100 mRNA targets (5).

We studied blood levels of three key microRNAs, miR-195, miR-21 and miR-155, in patients with breast cancer before surgery and also their changes post-mastectomy, in the frame of an ongoing project that is investigating the effect of mastectomy on kinetics of circulating novel biomarkers of breast cancer, especially those related to angiogenesis (6). The role of these microRNAs in cancer is well-established. miR-195 has been found to be overexpressed in both blood and tissue samples in patients with breast cancer and its level was higher in preoperative compared to postoperative samples (7). miR-155 has been shown to play a crucial role in fine tuning the regulation of lymphocyte immune function (8), to have a pivotal role in tumor angiogenesis in breast cancer (9), and be a key tumor-associated microRNA (oncomiR) overexpressed in multiple types of cancers, especially in breast cancer, with a prognostic significance (10-13). miR-21 is one of the most studied microRNAs in cancer (14). In breast cancer, high expression of miR-21 was correlated with poor survival (15) and, recently, it was found to be differentially expressed in early and late stages of this tumor type (16). To our knowledge, this is the first study to investigate these three key microRNAs in blood samples from patients with breast cancer, at diagnosis before surgery, and their postoperative kinetics.

Materials and Methods

Patients. Fifteen patients with breast cancer and five patients with breast fibroadenoma were enrolled in this study. The Institutional Review Board of the University Hospital of Ioannina approved the study protocol (2778/15.02.2008). Inclusion criterion was scheduled surgery for operable early breast cancer or benign tumor by the same team of breast surgeons. Exclusion criteria were: diabetes mellitus, active inflammation during the perioperative period, recent myocardial infarction, perioperative blood transfusion, erythropoietin administration and synchronous malignancy. Signed informed consent was obtained from all participating patients.

Correspondence to: Evangelos Briasoulis, MD, Ph.D., Professor of Oncology, University of Ioannina Medical School, Ioannina, 45110 Greece. Tel/Fax: +30 2651008087, e-mail: ebriasou@otenet.gr

Key Words: Breast cancer, biomarkers, miR-155, miR-195, miR-21.

Patient characteristics are shown in Table I. The patients with benign breast disease initially underwent an excisional biopsy in macroscopically healthy borders. If frozen section microscopy excluded cancer, a suction drain was placed and the operation terminated. All patients with breast cancer underwent core biopsy performed prior to surgery. The type of surgical procedure (modified radical mastectomy or wide local excision) was decided prior to surgery, following surgeon consultation and considering the patient's desire. The same experienced surgeon who led the Breast Unit performed all surgical procedures. A standard protocol of general anesthesia was applied to all cases, using propofol-fentanyl-muscle relaxant induction and O_2–Sevoflurane maintenance. A single pathologist who specializes in breast biopsies and immunohistochemistry carried out the histopathological examination of specimens. Patients with positive axillary lymph nodes received adjuvant therapy according to tumor biology, while those that underwent wide local excision also received adjuvant thoracic radiotherapy. Two patients developed tumor metastases within two years of surgery.

Acquisition of blood samples and storage. The same person drew blood *via* venipuncture for each sampling using a 21G BD Vacutainer Safety-Lok™ Blood Collection Set (BD Diagnostics, Franklin Lakes, NJ, USA). For each patient with breast cancer, four blood collections were obtained at the following time intervals: on the day before surgery and on postoperative days 3, 7 and 30. Samples from patients with fibroadenoma were collected preoperatively and on day 3 after surgery. All samples were collected in the morning (between 8 and 10 am) at room temperature. Each blood collection consisted of two PAXgene Blood RNA tubes (Preanalytix, GmbH, Hombrechtikon, Switzerland) containing 2.5 ml whole blood each.

PAXgene Blood RNA tubes were carefully incubated with the reagent and then placed in the upright position at room temperature for at least 2 hours (according to the manufacturer's recommendations), before being finally stored at −80˚C. All samples were stored frozen at the University of Ioannina Cancer Biobank Center.

RNA isolation. Total RNA was isolated using PAXgene blood RNA extraction kit (April 2011 version; PreAnalytix, GmbH) according to the manufacturer's instructions. The RNA quantity was measured with a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The RNA quality was estimated both by RNA gel electrophoresis 1.2% and Agilent Bioanalizer 2100 (Agilent Technologies, Waldbronn, Germany) (17). Only RNAs with an RNA integrity number greater than 7 were stored (−80˚C) and processed further.

Reverse transcription. For reverse transcription, miSCRIPT RT kit (Qiagen, Frederick, MA, USA) was used according to the manufacturer's protocol. Briefly, the same RNA quantity (0.5 μg) of all samples was initially polyadenylated and then reverse-transcribed. Concentrations of cDNAs were measured in a Nanodrop ND-1000 spectrophotometer. Then the samples were diluted with RNAse-free water to a final concentration of 0.5 μg/μl.

Quantification of miRNAs and statistical analysis. Quantification of the three microRNAs was performed following the miSCRIPT PCR Kit (Qiagen) protocol. Real-time polymerase chain reaction, qPCR, was carried out on a LightCycler 480 instrument (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany). A final 20 μl reaction mix was initially incubated at 95˚C for 15 minutes followed by a 3-step cycling, at 94˚C for 15 s, 55˚C for 30 s and 70˚C for 30 s (40 cycles). To normalize our results, *Caenorhabditis elegans* miR-39 was used as an external control since a specific number of miR-39 copies were added to the samples at the end of RNA extraction and before reverse transcription (18, 19). We quantified the results with the ΔΔCt method (18, 20). For statistical analysis, we used GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). Comparison of mean values of microRNA levels recorded over time in patients and between patients and controls was performed using the paired and unpaired t-test as appropriate. All tests were two-sided and considered significant when $p \leq 0.05$.

Table I. *Patients' demographics*.

	Benign breast disease	Breast cancer
Number of patients	5	15
Median (range) age, years	58 (35-76)	44 (41-47)
Disease	Fibroadenoma	Invasive ductal carcinoma
Disease site		
Right breast	3/5	11/15
Left breast	2/5	4/15
Surgical procedure		
WLE	5/5	
WLE + ALND	-	4/15
MRM + ALND	-	11/15
T Stage		
T1	-	6/15
T2	-	9/15
N Stage		
N0	-	7/15
N1	-	6/15
N2	-	2/15
Grade		
2	-	12/15
3	-	3/15
Hormonal receptor status		
ER^+	-	14/15
ER^-	-	1/15
PR^+	-	14/15
PR^-	-	1/15
HER2 status		
0	-	5/15
1	-	2/15
2	-	7/15
3	-	1/15
Blood samples	Pre, day 3	Pre, day 3,7,30

WLE: Wide local excision, ALND: axillary lymph node dissection, MRM: modified radical mastectomy, T: tumor, N: node, ER: estrogen receptor, PR: progesterone receptor; Pre: pre surgery.

Results

miR-195. Mean values of controls were not different on day 3 after surgery. In patients with breast cancer, the mean preoperative level of miR-195 was decreased on days 3 and

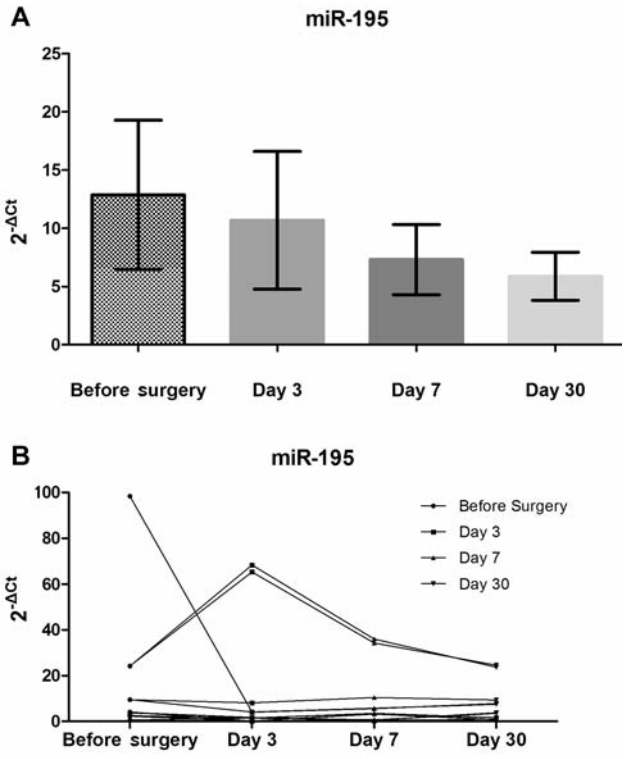

Figure 1. *Blood miR-195 levels in patients with breast cancer (n=15) before surgery and at three time points post-mastectomy. 1A. Data graphed as mean values with standard error of the mean. 1B. Data graphed as individual lines of kinetics of miR-195 levels of each patient. The two upper lines represent blood concentration of two patients, who eventually relapsed.*

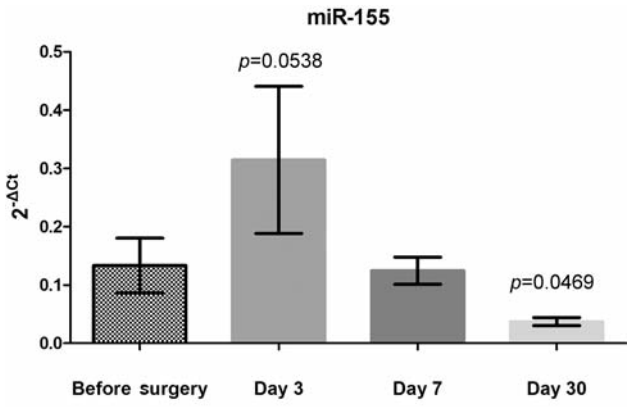

Figure 2. *Mean blood levels of miR-155 in patients with breast cancer (n=15) before surgery and at three time points post mastectomy. Data are graphed as mean values with standard error of the mean. p-Values are for comparison of data with baseline, before surgery, values.*

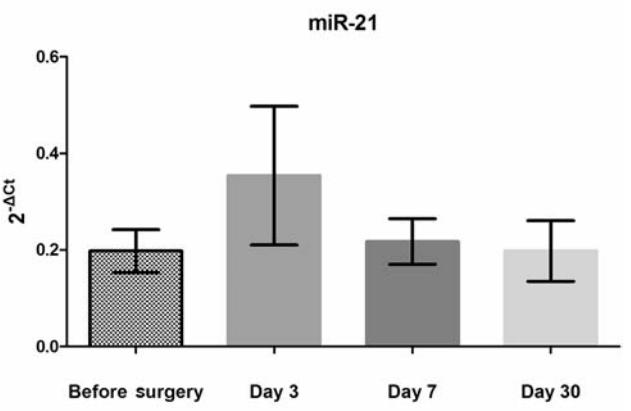

Figure 3. *Mean blood levels of miR-21 in patients with breast cancer (n=15) before surgery and at three time points post-mastectomy. Data are graphed as mean values with standard error of the mean.*

7 post-surgery but remained higher than that of controls even 30 days later, although changes did not reach statistical significance (Figure 1A). Concerning variation in miR-195 levels, the $2^{-\Delta Ct}$ values in patients with breast cancer ranged between 0.078 and 98.36 and in controls they were narrowly dispersed between 1.394 and 4.24. Interestingly two patients, who eventually experienced disease relapse, had the highest levels of miR-195 transcripts at all postoperative sampling times compared to other patients (Figure 1B).

miR-155. The mean miR-155 level of patients with breast cancer tended to be increased on day 3 after surgery (p=0.053). However, on day 7 the mean value had returned to the preoperative level and had fallen below baseline at day 30 (p=0.046) (Figure 2). The mean value for the controls also tended to increase on the third postoperative day (p=0.1348)

miR-21. Postoperative levels of miR-21 dropped significantly at three days post-surgery in the control arm (p=0.0001). In patients, the mean level of circulating miR-21 remained higher than that in controls throughout the sampling period but did not change significantly from baseline, and they did not follow any specific pattern of kinetics post-surgery (Figure 3).

Discussion

In the present study, we investigated baseline blood levels and postoperative kinetics of three key microRNAs, miR-155, miR-195 and miR-21, in patients with breast cancer who underwent mastectomy. The most striking findings in our study were i) the observation that two patients whose disease relapsed within two years had excessively high levels of miR-195 that remained steadily elevated for 30 days post-

surgery, and ii) the biphasic post-surgical kinetics of miR-155, which increased three days post-mastectomy and subsided below baseline values on day 30.

Of course these findings must be considered with regard to the small cohort of study patients, but to our knowledge, this is the first study to investigate the kinetics of three key microRNAs at multiple postoperative sampling times over a month's period post mastectomy.

It has been already reported that miR-195 might be used as a non-invasive diagnostic biomarker in early breast cancer. Heneghan *et al*. used a panel of seven microRNAs and found miR-195 and let-7a blood levels to be higher in patients with breast cancer compared to healthy individuals (21). In another study, the same investigators found that systemic miR-195 can be used to differentiate breast cancer from other malignancies and suggested that circulating miR-195 might potentially be used as a non-invasive biomarker for detecting early-stage breast cancer (7). We now provide for the first time an indication that persistently elevated levels of mIR-195 for at least one month post mastectomy may offer prognostic information regarding early tumor relapse. This finding deserves further evaluation in a large cohort of patients.

miR-155 has been found to be overexpressed in several cancer types (22). Regarding its implication in angiogenesis, Kong *et al*. suggested in a recent study that miR-155 has a pivotal role in tumor angiogenesis by inducing down-regulation of von Hippel-Lindau (9). In addition, Suarez *et al*. have shown that Vascular Endothelial Growth Factor induces the expression of miR-155 (23). Furthermore, by studying the impact of surgery on kinetics of miR-155, we provide for the first time indirect evidence that surgery may promote this angiogenesis-related microRNA, as reflected by its biphasic kinetics: an immediate increase of circulating miR-155 level that fall below baseline levels 30 days later.

miR-21 is one of the most extensively studied microRNAs in cancer. This microRNA is purported to act as an oncomiR and is up-regulated in human breast cancer (14, 24). In our work, the mean miR-21 level was marginally higher in patients with breast cancer compared to controls but this difference did not reach statistical significance. This microRNA probably has limited value in early breast cancer, considering that a high miR-21 level has been correlated with advanced clinical stage, lymph node metastasis and poor prognosis (25).

In conclusion, the findings of this pilot study indicate that miR-155 is possibly related to mastectomy-induced angiogenesis and that high postoperative levels of circulating miR-195 have potentially prognostic significance in patients with early breast cancer. Circulating miR-21 is of limited clinical importance in early breast cancer. Further investigation is warranted in a larger cohort of patients.

Acknowledgements

This research was co-financed by the European Union (European Social Fund – ESF) and Greek national funds through the Operational Program "Education and Lifelong Learning" of the National Strategic Reference Framework (NSRF) - Research Funding Program: Heracleitus II Investing in knowledge society through the European Social Fund.

References

1 Siegel R, Ma J, Zou Z and Jemal A: Cancer statistics. CA Cancer J Clin *64(1)*: 9-29, 2014.
2 Patani N, Martin L-A and Dowsett M: Biomarkers for the clinical management of breast cancer: International perspective. Int J Cancer *133(1)*: 1-13, 2013.
3 Harris L, Fritsche H, Mennel R, Norton L, Ravdin P, Taube S, Somerfield MR, Hayes DF and Bast RC Jr.: American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. J Clin Oncol *25(33)*: 5287-5312, 2007.
4 Uehara M, Kinoshita T, Hojo T, Akashi-Tanaka S, Iwamoto E and Fukutomi T: Long-term prognostic study of carcinoembryonic antigen (CEA) and carbohydrate antigen 15-3 (CA 15-3) in breast cancer. Int J Clinl Oncol *13(5)*: 447-451, 2008.
5 Bartel DP: MicroRNAs: genomics, biogenesis, mechanism, and function. Cell *116(2)*: 281-297, 2004.
6 Georgiou GK, Igglezou M, Sainis I, Vareli K, Batsis H, Briasoulis E and Fatouros M: Impact of breast cancer surgery on angiogenesis circulating biomarkers: a prospective longitudinal study. World J Surg Oncol *11(1)*: 213, 2013.
7 Heneghan HM, Miller N, Kelly R, Newell J and Kerin MJ: Systemic miRNA-195 differentiates breast cancer from other malignancies and is a potential biomarker for detecting noninvasive and early stage disease. The Oncologist *15(7)*: 673-682, 2010.
8 Seddiki N, Brezar V, Ruffin N, Levy Y and Swaminathan S: Role of miR-155 in the regulation of lymphocyte immune function and disease. Immunology *142(1)*: 32-38, 2014.
9 Kong W, He L, Richards EJ, Challa S, Xu CX, Permuth-Wey J, Lancaster JM, Coppola D, Sellers TA, Djeu JY and Cheng JQ: Upregulation of miRNA-155 promotes tumour angiogenesis by targeting VHL and is associated with poor prognosis and triple-negative breast cancer. Oncogene *33(6)*: 679-689, 2014.
10 Sun Y, Wang M, Lin G, Sun S, Li X, Qi J and Li J: Serum microRNA-155 as a potential biomarker to track disease in breast cancer. PloS One *7(10)*: e47003, 2012.
11 Gao F, Chang J, Wang H and Zhang G: Potential diagnostic value of miR-155 in serum from lung adenocarcinoma patients. Oncol Rep *31(1)*: 351-357, 2014.
12 Jurkovicova D, Magyerkova M, Kulcsar L, Krivjanska M, Krivjansky V, Gibadulinova A, Oveckova I and Chovanec M: miR-155 as adiagnostic and prognostic marker in hematological and solid malignancies. Neoplasma *61(3)*: 241-251, 2014.
13 Czyzyk-Krzeska MF and Zhang X: MiR-155 at the heart of oncogenic pathways. Oncogene *33(6)*: 677-678, 2014.
14 Krichevsky AM and Gabriely G: miR-21: a small multi-faceted RNA. J Cell Mol Med *13(1)*: 39-53, 2009.
15 Si H, Sun X, Chen Y, Cao Y, Chen S, Wang H and Hu C: Circulating microRNA-92a and microRNA-21 as novel

minimally invasive biomarkers for primary breast cancer. J Cancer Res Clinl Oncol *139(2)*: 223-229, 2013.
16 Ng EK, Li R, Shin VY, Jin HC, Leung CP, Ma ES, Pang R, Chua D, Chu KM, Law WL, Law SY, Poon RT and Kwong A: Circulating microRNAs as specific biomarkers for breast cancer detection. PloS One *8(1)*: e53141, 2013.
17 Briasoulis E, Aravantinos G, Kouvatseas G, Pappas P, Biziota E, Sainis I, Makatsoris T, Varthalitis I, Xanthakis I, Vassias A, Klouvas G, Boukovinas I, Fountzilas G, Syrigos KN, Kalofonos H and Samantas E: Dose selection trial of metronomic oral vinorelbine monotherapy in patients with metastatic cancer: a hellenic cooperative oncology group clinical translational study. BMC Cancer *13(1)*: 263, 2013.
18 Kroh EM, Parkin RK, Mitchell PS and Tewari M: Analysis of circulating microRNA biomarkers in plasma and serum using quantitative reverse transcription-PCR (qRT-PCR). Methods *50(4)*: 298-301, 2010.
19 Shi R and Chiang VL: Facile means for quantifying microRNA expression by real-time PCR. BioTechniques *39(4)*: 519-525, 2005.
20 Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(–Delta Delta C(T)) Method. Methods *25(4)*: 402-408, 2001.
21 Heneghan HM, Miller N, Lowery AJ, Sweeney KJ, Newell J and Kerin MJ: Circulating microRNAs as novel minimally invasive biomarkers for breast cancer. Ann Surg *251(3)*: 499-505, 2010.
22 Chira P, Vareli K, Sainis I, Papandreou C and Briasoulis E: Alterations of microRNAs in solid cancers and their prognostic value. Cancers *2(2)*: 1328-1353, 2010.
23 Suarez Y, Fernandez-Hernando C, Yu J,Gerber SA, Harrison KD, Pober JS, Iruela-Aripse ML, Merkenschlager M and Sessa WC: Dicer-dependent endothelial microRNAs are necessary for postnatal angiogenesis. Proc Natl Acad Sci USA *105(37)*: 14082-14087, 2008.
24 Si ML, Zhu S, Wu H, Lu Z, Wu F and Mo YY: miR-21-mediated tumor growth. Oncogene *26(19)*: 2799-2803, 2007.
25 Yan LX, Huang XF, Shao Q, Huang MY, Deng L, Wu QL, Zeng YX and Shao JY: MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. RNA *14(11)*: 2348-2360, 2008.

Received July 26, 2014
Revised September 11, 2014
Accepted September 18, 2014