Sindh Univ. Res. Jour. (Sci. Ser.) Vol. 47 (4) 687-692 (2015)

SINDH UNIVERSITY RESEARCH JOURNAL (SCIENCE SERIES)

MiRNAs as Potential Tumor markers for Early Diagnosis of Breast Cancer

F. NADEEM[++], Q. JAMAL*, M. HANIF**

Department of Pathology, Isra University, P.O Box 313, Hala Road, Hyderabad

Received 21st and 2014 and Revised 12th June 2015

Abstract: In females, the cancer of the Breast is the most oftenly diagnosed cancer. At present, miRNA emerges as potential tumor marker in many cancers including breast cancer (BC). These miRNAs are actually a specific group of short sequenced RNA which are non-coding. The present study was conducted to identify the level of expression of miR-497 in patients with breast cancer along with it's correlation with clinicopathologic features. A sample of 50 diagnosed cases of BC and 30 controls were selected. Blood samples were collected by venepuncture. Plasma was used for extraction of total RNA and cDNA was synthesized by using oligonucleotide primers for miRNA 497. Expression status of miR-497 was detected by RT-PCR. The results revealed that level of expression of miR-497 down regulated among 39 cases and 06 controls and had significant correlation with higher differentiation grade, metastasis in lymph node and late clinical stage (p= 0.001). Taken together, miR-497 might be used as possible marker of tumor for diagnosis as well as early detection of BC.

Keywords: Breast Cancer, miRNAs, tumor biomarker

1. INTRODUCTION

In the U.S (United States of America) cancer is the second most common cause of death. (Gilani *et al* 2010) Throughout the world, the cancer which is reported over and again in females is that of the breast. (Gilani *et al* 2010, Jemal *et al* 2011) Moreover it is one of the most important causes of mortality in women. (Sas *et al* 2014)

As reported by more than a few studies, Pakistani women have the highest risk of breast cancer among South Asian population, accounting for one third of all female cancers. (Forouzanfar *et al* 2011)

Moreover, it was also reported that breast cancer makes approximately 38% of all the cancers occurring in women referred to Nuclear Medicine, Oncology and Radiotherapy Institute [NORI], Islamabad, Pakistan. (Faheem *et al* 2007)

Furthermore in Pakistan studies about the prevalence of breast cancer reported that the possibility of having breast cancer is one out of nine women and is responsible for 40,000 deaths every year out of the 90,000 cases diagnosed. (Salman 2013)

The developed countries have cancer registry centres which register each patient who is diagnosed with cancer. This practice helps in understanding of eitiological factors and epidemiology of Breast cancer. The situation is different in underdeveloped countries such as Pakistan. Here we don't have any inclusive database or registry centre which provide the accurate number of the breast cancer patients reported. (Bhurgri *et al* 2006)

In developing countries the diagnostic resources are scarce and therefore large numbers of the females come to attention after the cancer has reached the advanced stage or has metastasized. These patients usually have poor outcome. (Gilani *et al* 2010) The scientific discoveries have reported that through early detection and management of these patients, the rate of survival and quality of life can be improved. (Montazeri *et al* 2008)

It is found that molecular diagnostic tests are not only important in diagnosis but early diagnosis and can be of help in planning specific and modified treatment strategies, hence decreasing mortality

MicroRNA

The discovery of microRNAs (miRNAs, miRs) in the 1990s, that have an overall inhibitory effect on expression of genes, opened a new window in understanding both normal and cancer development. (Liu. 2012)

These miRNAs are actually a specific group of short sequenced RNA which are non-coding. They are classically preserved through species. These RNAs are single-stranded comprising of 21–25 nucleotides which function in silencing of gene at post-transcriptional level. (Greene *et al* 2010)

[++]Corresponding author: F. NADEEM Email: farkhundanadeem@gmail.com
*Ziauddin Medical University, Karachi, 4/B Shahrah-e-Ghalib, Block 6, Clifton, 75600, Karachi, Pakistan
**Karachi Institute of Radiotherapy and Nuclear Medicine (KIRAN), Safoora Goth Off University Road , Karachi

A lot of studies have reported that miRNAs take part in many important human physiological and pathological processes which include: normal cellular growth, cellular apoptosis, development and carcinogenesis. (Farazi *et al* 2013)

The miRNAs which are related with cancer may be functioning as tumor suppressive or tumor promotive (oncogenic) . (Profumo *et al* 2013)

Many miRNAs are related with BC and their expression levels are deregulated as reported by many studies, like Iorio and his colleagues who used microarray as well as Northern blot analyses. The author has further reported that the normal and tumor tissues could be noticeably distinguished through the level of expression of miRNAs. The most notably deregulated miRNAs are mir-21, mir-125b, mir-145, and mir-155. (Iorio *et al* 2005)

The miRNAs are remarkably stable in fluids present in body such as serum and plasma and could be easily detectable. This property make them a possible candidate for using these as noninvasive biomarkers for detection of cancers including breast cancer. (Cortez *et al* 2012). A small number of studies have been conducted about the possible role of miRNAs for early detection of BC. We have selected miR-497 for the present study. This miRNA is reported to be downregulated in many cancers like carcinoma of colon, cervix and prostate. (Luo *et al* 2013)

According to a genome-wide analysis for assessing the level of expression of miRNAs, following were found to be significantly downregulated in breast cancer: hsa-miR-497, hsa-miR-355, hsa-miR-31, hsa-miR-320, hsa-miR-127, rno-mir-140 and hsa-miR-30a-3p. (Yan *et al* 2008).

In our study, the level of expression of miR-497 was detected through qRT-PCR assay in patients with breast cancer as well as in controls. Moreover, the association of level of expression of miR-497 with various clinicopathologic features in same patients were also analyzed. Our results revealed that miR-497 was considerably down regulated in plasma of BC patient and can be used as a probable non-invasive molecular biomarker for detection of BC in early stage.

2. METHODS AND MATERIALS

Study population and blood samples

Study population consists of females with breast cancer and controls (healthy/and non-cancerous patients). The data was collected from Liaquat University Hospital (LUH), Jamshoro, Isra University Hospital Hyderabad and Karachi Institute of Radiotherapy and Nuclear Medicine (KIRAN), Karachi . The women were selected regardless of age, parity and social class. Patients suffering from any other cancer beside breast cancer were not included in the study.

After obtaining written informed consents from all study participants a total of 139 peripheral blood samples from the breast carcinoma patients and 70 from controls (healthy or non-cancerous patients) was collected in EDTA bottles and was transported to laboratory in ice box to maintain the temperature for RNA integrity. In the laboratory the sample bottles were allowed to stand for 2 hours in refrigerator. Then plasma was aspirated with the help of justure and small disposable tips from each EDTA bottle in labeled 5 ml appendorf tubes and stored at -40^0c till the procedure was performed for extraction of RNA.

Patient's personal information, relevant history, histological diagnosis and grading of the tumors were recorded from the laboratory reports and clinical finding were noted from the hospital record of the patients.

Total RNA Extraction

Total RNA (including MiRNAs) from all the plasma samples were extracted by TRIZOL LS method according to the protocol provided by the suppliers (invitrogen: Cat. No. 10296-028) as described previously . (Chomczynski *et al* 1987)

Detection of level of expression of miR-497 through quantitative reverse transcription (qRT)-PCR

Total RNA (5 µl) was used for synthesis of the cDNA , as per instructions of protocol provided by Thermo Scientific, RevertAid First Strand cDNA Synthesis Kit M/s #K1622 (Fermentas). For the procedure oligonucleotide primers for miRNA – 497 were used (synthesized by IDT (INTEGRATED DNA TECHNOLOGIES).

PCR was done by using ThermoScientific Maxima SYBER green/ROX qPCR Master Mix (2x) by Thermo Scientific (# K0221) according to the instruction as per suppliers.

Amplification in the form of CT (Threshold cycle) was noted and quantification was done by ΔΔCt cycle threshold method after normalization to that of U6 (used as internal control) .

Statistical analysis

The data was analyzed through SPSS version 21.0 for windows release (IBM, incorporation, USA) . Student's t-test and Chi-square test were used to assess continuous and categorical variables. Mean ± SD was use to present continuous variables while frequency and percentages were used for categorical variables. We

used Microsoft excel for making graph. The results are shown in graphs and tables. *P* value of ≤ 0.05 was taken as significant.

3. RESULTS

Expression of miRNA 497

The level of expression of miR-497 was find out by qRT-PCR assay in 50 patients with BC and 30 healthy or non-cancerous controls.

The results showed that out of 50 plasma samples from cases of BC, the level of expression of miR-497 was down regulated in 39 cases and upregulated in only 11 cases of BC. **(Table 1).**

Table 1 Expression of miR-497 in BC patients and Controls

miR-497 expression		
	BC patient (n=50)	Controls (no=30)
Higher	11	24
Lower	39	06

Correlations of level of expression of miR-497 with various clinicopathologic features in patients with BC

Then the relationship of expression status of miR-497 with various clinicopathologic features of same 50 BC patients was found out and analyzed statistically. According to the results, it was found that there is noticeably significant relationship of low miR-497 expression with higher grade and spread of tumor to lymph node and advanced clinical stage with p value of 0.0001, 0.0001 and 0.0001 respectively .(table 2) Nevertheless, it was also found that there is no noteworthy relationship among the level of expression of miR-497 and rest of the clinicopathologic parameters like age, history of taking exogenous hormones , history of breast cancer in 1st degree relative and tumor size with p value P = 0.09, 0.06, 0.05, and 0.07 respectively. **(Table 2)**

Table 2 Relationship of miR-497 expression status with clinicopathologic features of BC Patients

Clinicopathologic features	High (n=11)	Low (n=39)	p- value
Age (years)			
≤50 (44)	06	38	0.09
>50 (06)	-05	01	
H/O taking exogenous hormones			
Yes (19)	04	15	0.06
No (31)	07	24	
H/O breast cancer in 1st degree relative			
Yes (16)	05	11	**0.05**
No (34)	06	28	
Tumor size (cm)			
≤2.0 (12)	06	05	0.07
>2.0 (38)	05	33	
Differentiation grade			
G1 + 2 (42)	09	33	**0.0001**
G3 (08)	02	06	
Lymph node metastasis			
Absent (21)	04	17	**0.0001**
Present (29)	07	22	
Clinical stage			
I + II (31)	06	25	**0.0001**
III+ iv (19)	05	14	

4. DISCUSSION

In both developed and under developed countries, cancer of the breast (BC) has reached the top most position amongst the cancers in women. (Wang *et al* 2013)

Moreover, in the year 2013, American Cancer Society has reported 232,340 cases of registered BC in women and around 39,620 females died because of it in the United States. Therefore, it was expected that 2013, the incidence of BC in women accounts 29% . (Siegel *et al* 2013).

Regardless of the high prevalence and death ratio connected to BC, early detection of it is still not possible because of the lack of awareness programs like the importance of self-examination of breast and screening programs like mammography. This may be responsible for diagnosis of the cancer in late stage resulting in increased mortality. (Badar *et al* 2007)

MiRNAs signify its impending role as biomarkers, which are non- invasive and can help in early diagnosis as well in prognosis of many cancers. (Shen *et al* 2013)

A lot of researches about cancers, have supported the importance of the likely function of miRNAs as biomarkers in many human cancer including BC. (Weigel *et al* 2010)

Increased expression of miR-210 may be related with poor survival in BC patients as reported by Hong' *et al* in a meta-analysis. (Hong *et al* 2012)

MiRNAs are helpful not only in diagnosis of cancers but also in their prognosis as reported by Tang and colleagues in 2012. They have reported that increased expression of miR-27 in women with breast cancer was related with overall poor survival, signifying that miR-27 could be an important marker of progression of carcinoma of breast. (Tang *et al* 2012)

Gene for miR-497 is present at chromosome 17p13.1. It reveal a tumor suppressive effect in a

number of cancers, like hepatocelluar carcinoma, cancer of colon, neroblastoma, non- small cell cancer of lung and breast cancer . (Zhao *et al* 2013, Furuta *et al* 2013, Creevey *et al* 2013)

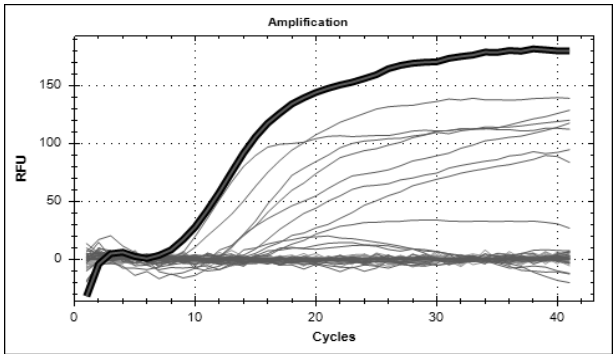
It is reported by Zhou *et al* that miRNA-9 might be used as a possible biomarker for detection of local recurrence of cancer of breast and level of the estrogen receptors by analyzing expression status of 754 human miRs in tissues from16 breast cancer. Out of these, 8 cases were with local recurrence while 8 cases were without local recurrence. (Zhou *et al* 2012),

These studies show that the expression miRNAs are not only helpful in diagnosis but their expression status is also cancer specific and therefore can be used as a potential biomarker for cancers.

Keeping all these facts in mind this study was conducted with the aim to investigate the expression status of miRNA 497 in BC patient and in controls using their plasma and to find out correlations of the same with clinicopathologic features in the patients studied. The results revealed that the relative expression of miR-497 in patients with BC was considerably less than in controls. These findings are supported by the results of Dan Li *et al* who reported that level of expression of miR-497 and miR-195 are inversely related with malignant tumor of breast. Such expression pattern could potentially recognize cancer from benign tumors or normal. (Li *et al* 2011) There have been very few studies on the correlation of miR497 expression level and various clinicopathologic parameters. Therefore, the correlations of the level of expression of miR-497 with various clinicopathologic parameters was found. The results showed that expression status of miR-497 in plasma samples of women with BC was considerably correlated with tissue differentiation grade, metastasis of tumor to lymph node and clinical stage of patients with BC. The results also revealed that these BC patients (with low miR-497 expression) showed poor histological grade, increased incidence of metastasis to lymph node and higher clinical stage. These results suggest that downregulation of miR-497 play a significant role in progression of BC. It was also found that although low level of miR-497 is notably linked with advanced clinical stage but its expression start decreasing from the stage II and even in stage I and this property make it a potential marker for diagnosis of BC in early stages.

Therefore, it can be concluded from the present study that miR- 497 remain stable in body fluids like serum and plasma provided the temperature required for their integrity is maintained. Its expression starts decreasing from stage I of BC and because of this property they might be used as an independent non-invasive potential molecular biomarker not only for diagnosis but also for early detection of breast cancer.

Certainly, the present study has few limitations. The number of cases were less, especially those having stage I and stage II (as the samples were collected randomly from BC patients) and a larger case population study comprising of equal number of all stages is required to validate the value of miR-497 and its level of expression in patients with BC for diagnosis of disease in early stages.



Graph: qRT-PCR detection of relative miR-497 expression (Amplification and down regulation) in controls and BC cases (One batch: BC =20, Controls = 20)

REFERENCES:

Badar, F., Z. S. Faruqui, A. Ashraf, and N. Uddin, (2007) Third world issues in breast cancer detection. *The Jour. of the Pakistan Medical Association.* 57: 137-139

Bhurgri, Y., A. Bhurgri, S. Nishter, A. Ahmed, A. Usman, S. Pervez, N. Kayani, R. Ahmed, S. H. Hassan, and A. Riaz, (2006) Pakistan-country profile of cancer and cancer control 1995-2004. *Journal of the Pakistan Medical Association.* 56: 124-130.

Chomczynski, P., and N. Sacchi, (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical biochemistry.* 162**:** 156-159.

Cortez M A., J. W. Welsh, and G. A. Calin (2012) Circulating microRNAs as noninvasive biomarkers in breast cancer. *Recent Results Cancer Res.* 195:151–161.

Creevey, L., J. Ryan, H. Harvey, I. M. Bray, M. Meehan, A.R. Khan, and R. L. Stallings, (2013) MicroRNA-497 increases apoptosis in MYCN amplified neuroblastoma cells by targeting the key cell cycle regulator WEE1. *Mol Cancer.* 12**,** 23Pp.

Faheem, M., M. Khurram, I. A. Jafri, H. Mehmood, Z. Hasan, G. S. Iqbal, F. Maqsood, and S. R. A. Jafri,

(2007) Risk factors for breast cancer in patients treated at NORI Hospital, Islamabad. *JPMA. The Journal of the Pakistan Medical Association*. 57:242-245.

Farazi, T. A., J. I. Hoell, P. Morozov, and T. Tuschl, (2013) MicroRNAs in human cancer. *MicroRNA Cancer Regulation.* Springer. *Advances in Experimental Medicine and Biology*. 774:1–20.

Forouzanfar, M. H., K. J. Foreman, A. M. Delossantos, R. Lozano, A. D. Lopez, C. J. Murray, and M. Naghavi, (2011) Breast and cervical cancer in 187 countries between 1980 and 2010: a systematic analysis. *The Lancet.,* 378**:** 1461-1484.

Furuta, M., K. I. Kozaki, K. Tanimoto, S. Tanaka, S. Arii, T. Shimamura, A. Niida, S. Miyano, and J. Inazawa, (2013) The tumor-suppressive miR-497-195 cluster targets multiple cell-cycle regulators in hepatocellular carcinoma. *PLoS ONE.,* 8:1-12. e60155.

Gilani, S. I., M. Khurram, T. Mazhar, S. T. Mir, S. Ali, S. Tariq, and A. Z. Malik, (2010) Knowledge, attitude and practice of a Pakistani female cohort towards breast cancer. *JPMA. The Journal of the Pakistan Medical Association.*, 60: 205.

Greene, S. B., J. I. Herschkowitz, and J. M. Rosen, (2010) Small players with big roles: microRNAs as targets to inhibit breast cancer progression. *Current drug targets.* 11**:** 1059-1073.

Hong, L., J. Yang, Y. Han, Q. Lu, J. Cao, and L. Syed, (2012) High expression of miR-210 predicts poor survival in patients with breast cancer: a meta-analysis. *Gene.* 507**:**135-138.

Iorio, M. V., M. Ferracin, C. G. Liu, A. Veronese, R. Spizzo, S. Sabbioni, E. Magri, M. Pedriali, M. Fabbri, and M. Campiglio, (2005) MicroRNA gene expression deregulation in human breast cancer. *Cancer research.* 65**:**7065-7070.

Jemal, A., F. Bray, M. M. Center, J. Ferlay, E. Ward, and D. Forman, (2011) Global cancer statistics. *CA: a cancer journal for clinicians.* 61**:**69-90.

Leen, S., V. L. Steven, D. A. Marie, D. Valérie, D. P. Annemie, V. D. E. Gert, V. D. Peter, D. Luc, V. Peter, and L. Filip, (2014) Leen, S., V. L. Steven, D. A. Marie, D. Valérie, D. P. Annemie, V. D. E. Gert, V. D. Peter, D. Luc, V. Peter, and L. Filip, (2014) Study Assessing the Quality of Quantification of Estrogen Receptor Protein Expression by Immunohistochemistry and Gene Expression in Breast Cancer. *Pathology research international.* 2014:1-8.

Li, D., Y. Zhao, C. Liu, X. Chen, Y. Qi, Y. Jiang, C. Zou, X. Zhang, S. Liu, and X. Wang, (2011) Analysis of MiR-195 and MiR-497 expression, regulation and role in breast cancer. *Clinical Cancer Research.* 17**:**1722-1730.

Liu, H., (2012) MicroRNAs in breast cancer initiation and progression. *Cellular and Molecular Life Sciences.* 69**:** 3587-3599 .

Luo M., D. Shen, X. Zhou, X. Chen, and W. Wang, (2013) MicroRNA-497 is a potential prognostic marker in human cervical cancer and functions as a tumor suppressor by targeting th insulin-like growth factor 1 receptor. *Surgery*. 153:836–847.

Montazeri, A. (2008) Health-related quality of life in breast cancer patients: a bibliographic review of the literature from 1974 to 2007. *Journal of experimental & clinical cancer research.*27**:** 32Pp.

Profumo, V., and P. Gandellini, (2013) MicroRNAs: cobblestones on the road to cancer metastasis. *Critical Reviews™ in Oncogenesis.*18.

Shahed, S., (2013) The Impact of Health Information Provision on Breast Cancer-Related Knowledge and Protective Behaviors: An Experiment in Health Education. *Lahore Journal of Economics*.18**:**117-133.

Shen, J., S. A. Stass, and F. Jiang, (2013) MicroRNAs as potential biomarkers in human solid tumors. *Cancer letters.* 329**:**125-136.

Siegel, R., D. Naishadham, and A. Jemal, (2013) Cancer statistics, 2013. *CA: a cancer journal for clinicians.* 63**,** 11-30.

Tang, W., J. Zhu, S. Su, W. Wu, Q. Liu, F. Su, and F. Yu, (2012) MiR-27 as a prognostic marker for breast cancer progression and patient survival. *PLoS ONE.* 7**:**1-7**.** e51702.

Wang, S., H. Li, J. Wang, and D. Wang, (2013) Expression of microRNA-497 and its prognostic significance in human breast cancer. *Diagn Pathol.* 8:172-178.

Weigel, M. T., and M. Dowsett, (2010) Current and emerging biomarkers in breast cancer: prognosis and prediction. *Endocrine-related cancer.* 17: R245-R262.

Yan, L. X., X.. F. Huang, Q. Shao, M.Y. Huang, L. Deng, Q. L. Wu, Y. X. Zeng, and J. Y. Shao, (2008) Micro RNA miR-21 over expression in human breast cancer is associated with advanced clinical stage, lymph

node metastasis and patient poor prognosis. *RNA.*14**:**2348-2360. . 14**:**2348-2360.

Zhao, W. Y., Y. Wang, Z. J. An, C. G. Shi, G. A. Zhu, B. Wang, M. Y. Lu, C. K. Pan, and P. Chen, (2013) Down regulation of miR-497 promotes tumor growth and angiogenesis by targeting HDGF in non-small cell lung cancer. *Biochemical and biophysical research communication.* 435**:** 466-471.

Zhou, X., C. Marian, K. H. Makambi, O. Kosti, B. V. Kallakury, C. A. Loffredo, and Y. L. Zheng, (2012) MicroRNA-9 as potential biomarker for breast cancer local recurrence and tumor estrogen receptor status. *PLoS ONE.* 7: 1-9.