miR-16: A novel hereditary marker in breast cancer and their offspring
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Abstract
Objective: To analyse micro ribonucleic acid-16 in sera of invasive intraductal breast carcinoma in stage III and compare its expression in their daughters and healthy women.
Methods: The study took place from January 2013 till December 2015. This case-control study was conducted at the Ziauddin Cancer Hospital, Karachi, and comprised breast cancer patients and healthy individuals. Stage III invasive intraductal breast cancer patients (cases), their age-matched healthy individuals (control group A) and patients' daughters (control group B) were included. Subjects with stage I cancer and their daughters and subjects with stage IV and their daughters were also included. Serum tests were run on real-time quantitative reverse transcription polymerase chain reaction. Threshold cycle was determined and fold change was calculated. Fold change was applied between the groups. SPSS 20 was used for data analysis.
Results: Of the 194 participants, there were 50 (25.8%) cases, 50 (25.8%) group A controls, 35 (18%) group B controls, 20 (10.3%) stage I patients, 11 (5.7%) daughters of stage I patients, 20 (10.3%) patients of stage IV and 8 (4.1%) daughters of stage IV patients. Micro ribonucleic acid-16 was higher in cases than controls (p=0.001). Group B showed significant gene expression than group A (p=0.001). Stage IV patients and daughters showed expression of micro ribonucleic acid-16 (p=0.001). Triple negative receptor cases showed a greater expression of gene (p=0.001).
Conclusion: Micro ribonucleic acid-16 can be used as a prognostic, diagnostic as well as a predictive marker in breast cancer patients and their offspring.
Keywords: MiR-16, Intraductal, Breast carcinoma, ER, PR, HER2, Triple negative. (JPMA 67: 446; 2017)

Introduction
Micro ribonucleic acid (RNA) - or miR - is found in all living organisms and are considered as small single-stranded RNAs made up of 18-20 nucleotides which are derivatives of post-transcription. These miRs have the ability to bind to 3′untranslated region (UTR) of the targeted mRNA.1 If these miRs undergo dysregulation, they may increase oncogenes or suppress tumour suppressor genes resulting in cancerous change at cellular level. Therefore, formation of anti-miR molecules may be a promising therapy for cancerous disorders. An miR-16 is one of the identified microRNAs that play a role in breast cancers; its location is in 13q14 chromosome and its mode of action is on the cell cycle. It is involved in regulating gene expression that may be implicated in cancer development. It is under research that expression of RNA binding protein human antigen R (HuR) in breast cancer causes up-regulation of cyclin E1 but other researches show miR-16 represses cyclin E1.2

The ubiquitous miR-16 is one of the first identified microRNAs that are involved in the initial stage of cancers which also causes progression and metastasis of cancer cells. Generally, miR-16 is considered to be down-regulated in many types of cancers, thus considered to behave as tumour suppressor gene which has the ability to target numerous oncogenes of different origins of cancer. MiR-16 is shown to promote apoptosis of cancer cells by effecting FEAT (faint expression in normal tissues, aberrant over-expression in tumours). It is clearly declared in a study by Stuckrath et al. that plasma levels of miR-16 were significantly higher in women suffering from breast cancer before the intervention of chemotherapy and this microRNA was reduced remarkably after treatment of the malignancy if it had been eradicated from an individual.3

The protein receptors of breast cancer indicate the prediction and prognosis of breast cancer, patients with positive receptors undergo endocrine adjuvant therapy along with chemotherapy. The testing of oestrogen receptor (ER) and progesterone receptor (PR) is carried in all patients suffering from breast cancer as a routine test.4 Therapy that is used for inhibition or in reducing the effect of ER-activated pathways includes oestrogen receptor modulators, for example tamoxifen and aromatase inhibitors that is arimidene. Another type of protein receptor that is routinely sent for investigation is human epidermal growth factor receptor 2 (HER2) and therapy to combat this protein includes trastuzumab and lapatinib.5 However, several studies in the past decade have proven that oestrogen and progesterone tumours may adapt severe
resistance to the underlying treatment and the cancer of the breast may continue to undergo progression and even metastasis is likely to occur. The common methodology to determine miRs in various cases is ideally by northern blot analysis, but its drawback is that this procedure requires an increased quantity of blood, approximately 100ml, for proper results. A more convenient method that is being undertaken by researchers is real-time polymerase chain reaction (RT-PCR) and microarrays, but these are being conducted at research level to date due to its expense; it has not yet been used routinely as an investigation in commercial labs. These microRNAs are shown to play a role in normal cellular functions which includes proliferation, differentiation and apoptosis but if dysregulation of this gene expression occurs and it is deviated from its normal pathway, it may result in devastating diseases such as malignancies and other disorders.

These are, therefore, a class of gene expressed aberrantly and may also undergo mutation resulting in cancers and are novel markers that may behave as oncogenes or tumour suppressor genes.

The current study was planned to analyse circulating miR-16 in sera of invasive intraductal breast carcinomas of stage III, compare its expression in daughters of the index cases as well as in healthy women, and to compare serum miR-16 between stage I and IV of intraductal breast cancer and daughters of such patients.

**Patients and Methods**

The study took place from January 2013 till December 2015. This case-control study was conducted at the Ziauddin Cancer Hospital, North Nazimabad, Karachi, Pakistan, and comprised breast cancer patients and healthy subjects. Patients suffering from breast cancer of invasive ductal category in stage III (cases), their age-matched healthy controls (control group A), daughters of index cases (control group B) were included. Patients suffering from stage I and their daughters, and patients of stage IV and their daughters were also included.

Controls A were recruited by purposeful sampling and were disease-free. Controls B were recruited if they were not suffering from breast cancer or any other cancer.

Subjects with invasive ductal carcinoma were recruited after histological confirmation of biopsy reports. Subjects visiting the oncology clinic were randomly picked who had not undergone any chemotherapy or surgery; they were newly diagnosed patients with breast carcinoma, with their biopsy reports showing results of ER, PR and HER2. The daughters of these patients were given appointments to visit the cancer hospital and were invited to be a part of this study after providing informed consent.

Then 5ml blood was drawn after obtaining written informed consent from cases (stage I, III, IV) controls A, B of stage III and daughters of stage I and IV. The blood was collected in a BD (Becton Dickinson) gel vacutainer (yellow top), it was centrifuged at 3,000 revolutions per minute (rpm). Serum was extracted and transferred to a conical bottom tube. Around 3ml serum was obtained which was stored at -80°C. The real-time quantitative reverse transcriptase (qRT) PCR was used for the experiment (make: Rotor-Gene Q, SN R-060953 Qiagen Hilden, Germany).

For RNA elusion miRN easy serum/plasma spike-in control which consisted of 10pmol Caenorhabditis (C.) elegans miR-39 miRNA mimic spike-in control for serum samples was considered as a standardised positive control for all recruited subjects. After RNA elusion, complementary deoxyribonucleic acid (cDNA) preparation was done; for this, miScript RT II kits were used by utilising reagents for cDNA synthesis reactions. The cycling conditions were set at 40 cycles and the procedure for real-time PCR were 15 seconds at 94°C for denaturation, 30 seconds at 55°C for annealing and 30 seconds at 70°C for extension.

The reaction set up for RT-PCR started by using miScript SYBR (Synergy Brands inc) stock brand, Green PCR kit and miScript primer assay C.elegans miR-39 was considered as positive control (pc) and reference for miR-16 primer. This miScript primer assay has binding affinity to spike-in control showing certain amplification and gene expression. Thus, this was used as a standard for it showed gene expression in both cases and controls. With reference to this the fold change was calculated.

The first reaction for miR-16 was sequence 5'tagcagcagtaaatagcg3'. All the reactions were done in duplicate. The threshold cycle (Ct) is the number of cycles at which the fluorescence passes at a pre-determined threshold. Expression analysis was done to compare cases with controls.

The relative quantification of serum miR-16 in all three groups was calculated using the equation; amount of target= 2^((Ct pc - Ct miR-16)- (Ct mean pc - Ct mean miR-16)). In order to determine the gene expression the fold change for all groups was calculated. Melting curves were generated and RT-PCR amplification was performed. Moreover, miR-16 were extracted from the circulation and compared between the groups and with the status of ER, PR and HER2.

Microsoft Excel 2007 was used for data entry. SPSS 20 was used for data analysis. Frequency and percentages were used to express outcome of categorical variables. Chi-square test was used to test association among the types of patients and their categorised clinical findings. Fisher’s exact test was used while analysing the same between cancer
patients and healthy patients or with their daughters. Descriptive measures for symmetric continuous variable were expressed as mean with its standard deviation (SD). For skewed continuous variables, median with inter-quartile range was calculated. Normality of continuous variables was assessed by Shapiro-Wilk's test. One way analysis of variance (ANOVA) and Kruskal-Wallis test was used to compare symmetric and skewed continuous variables, respectively, among the groups. While comparing the same between two groups, Student's t-test and Mann-Whitney U test was used. Graphs were constructed to display the continuous variable measures. P<0.05 was considered significant.

**Results**

Of the 194 participants, there were 50 (25.8%) cases, 50 (25.8%) group A controls, 35 (18%) group B controls, 20 (10.3%) stage I patients, 11 (5.7%) daughters of stage 1 patients, 20 (10.3%) patients of stage IV and 8 (4.1%) daughters of stage IV patients. Overall, there were 90 (46.4%) cancer patients. The mean age of the cancer participants was 43±10.5 years.

Among stage III infiltrative ductal carcinoma patients, ER+ve was found in 16 (32%) and ER-ve in 18 (36%). Tissue with PR+ve were present in 17 (34%) participants and PR-ve in 17 (34%) patients; HER2 was positive in 17 (34%) and negative in 17 (34%) (Table-1).

Right side was involved in most of them 31 (62%). Breast cancer was found in the family history of 14 (28%) cancer patients, all from the maternal side.

The mean Ct value of miR-16 was 31.37±7.6 in cancer patients. Expression of miR-16 was positive in 34 (68%) patients, 12 (34%) in group B (high-risk population) and 10 (20%) in group A. The cancer patients showed the highest expression, however, the mean miR-16 was significantly higher in group B as compared to group A (p<0.0001).

We also divided the group into two sub-groups i.e. those with stage I carcinoma and stage IV carcinoma. It was determined that miR-16 expressed was weak in stage I patients but strong in stage IV. The daughters of stage I showed no expression whereas daughters of stage IV showed expression of miR-16. The mean age of stage IV patients was 58±3.1 years and their daughters was 41.1±3.22 years. The fold change expression was determined, showing significant expression of miR-16 in breast cancer patients and their daughters compared to normal healthy individuals who showed no expression of miR-16 (Figure: a and b).

It was determined that miR-16 expressed was weak in stage I patients but was strong in stage IV patients. In the daughters of stage I patients there was no expression whereas daughters of stage IV patients showed expression of the gene. The mean age of patients of stage I cancer and their daughters were 36±2.5 years and 19±4.2 years, respectively (Table-2).

**Table-1:** The number of cancer patients whose serum miR-16 showed significant Ct values and Foldchange.

<table>
<thead>
<tr>
<th>Proteins detected in breast biopsy tissue</th>
<th>MicroRNA-16 in serum of breast cancer stage III patients. Total patients: n = 50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Receptors</td>
<td>Ct value</td>
</tr>
<tr>
<td>------------------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>ER +ve</td>
<td>16 (32)</td>
</tr>
<tr>
<td>ER -ve</td>
<td>18 (36)</td>
</tr>
<tr>
<td>PR +ve</td>
<td>17 (34)</td>
</tr>
<tr>
<td>PR -ve</td>
<td>17 (34)</td>
</tr>
<tr>
<td>HER2 +ve</td>
<td>17 (34)</td>
</tr>
<tr>
<td>HER2 -ve</td>
<td>17 (34)</td>
</tr>
<tr>
<td>Triple Negative</td>
<td>12 (24)</td>
</tr>
</tbody>
</table>

RNA: Ribonucleic acid
ER: Oestrogen receptor
PR: Progesterone receptor
HER2: Human epidermal growth factor receptor 2.

**Table-2:** Comparison of elderly patients and their daughters with stage I and IV intra ductal breast cancer.

<table>
<thead>
<tr>
<th>Investigations</th>
<th>Stage I (n=20)</th>
<th>Stage IV (n=20)</th>
<th>Daughters of stage I (n=11)</th>
<th>Daughters of stage IV (n=8)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-16-Ct values</td>
<td>45.16±5.52</td>
<td>26.66±3.56</td>
<td>negative</td>
<td>30.2±6.87</td>
<td>0.001</td>
</tr>
<tr>
<td>Triple negative</td>
<td>49.32±4.98</td>
<td>30.77±6.63</td>
<td>44.88±5.71</td>
<td>31.78±2.13</td>
<td>0.001</td>
</tr>
</tbody>
</table>

miR: Micro ribonucleic acid
Ct: Threshold cycle.


Discussion

Based on reverse transcriptase real-time PCR, our study findings quantified miR-16 in the serum of subjects of breast cancer patients after confirmation by biopsy report. MicroRNA-16 has been quantified by other studies and most importantly investigators have used miR-16 as a normalisation control. The levels of miR-16 were measured by determining the Ct value and fold change. This was measured in serum of breast cancer patients suffering from mostly stage III intraductal invasive carcinoma of the breast and were found to be expressed significantly higher than in healthy women showing the diagnostic potential of this miR in such cases of breast cancer. Stuckrath et al. showed similar changes in plasma levels of Indian patients suffering from breast cancer. These authors also showed that miR-16 was increased in lymph-node negative patients which was not apparent in our findings. In various studies, this well-studied gene miR-16 has been described as an oncogene, tumour suppressor gene or as a control reference.

Interestingly, miR-16 expression has also been reported as a potential therapeutic target and clinical biomarker. In cases of breast cancer where bone metastasis has also taken place, it is increased in osteoclast differentiation and bone metastasis. Our research showed that ER+ve, PR+ve and HER2+ve cancers showed negative fold change values which may be interpreted as down-regulation of miR-16 in such patients; however, up-regulation of foldchange was calculated in ER-ve, PR-ve and HER2-ve. Tissues having triple negative protein receptors showed up-regulation indicating that the disease is more devastating in triple negative protein receptors with poor prognosis. Zhang et al. also confirmed the up-regulation in triple negative cases. Moreover, they also detected that chemotherapy had no effect in decreasing the miR-16 levels in postoperative plasma of the breast cancer patients. This could be explained by the inflammatory processes which release increasingly miR-16 into the blood circulation.

We also studied the serum miR-16 levels in daughters of the recruited breast cancer patients (stage III) who are potentially the high risk-group of women. Literature shows that women whose parents or grandparents have had breast cancer, may inherit these genes. It was determined that 34% of the daughters showed enhanced expression of miR. Foldchange of miR in daughters was also determined which showed up-regulation as compared to the healthy controls. No study to date has been done on daughters and this study is a small indicator of the predictive potential of the breast cancer in the population who are relatively at a high risk than others.

Mobarrat et al. reported that over-expression of miR-16 declines cellular growth and proliferation, and induces apoptosis in human cancer which is contradictory to our findings and other earlier reported studies. These authors declared that manipulation of this single-stranded RNA molecule found in all multi-cellular organisms can be a promising approach in cancer therapy and experimental results represented that the modification in breast cancer phenotype is possible by miR expression alteration. They reported that miR-16, which is located in 13q14 chromosome, plays a critical role as a tumour suppressor by targeting several oncogenes which regulate cell cycle and apoptosis.

In our study, miR-16 was found in all groups and it may be hypothesised that instead of this gene being the cause of the disease it is resulting in stimulating tumour suppressor gene (TSG) and by the mechanism of apoptosis causes cellular death. This gene may also be present in other stages of breast cancer as it can be seen that those women with stage IV cancer have a greater expression of the gene and such individual’s offspring may also be labelled as high-risk groups. These miRs in the advanced stage may also migrate to osteoclast where they may produce a beneficial effect but its mechanism of action is still unclear to date. Evidence indicates that miR-16 can modulate the cell cycle, inhibit cell
proliferation, promote cell apoptosis and suppress tumorigenicity both in vivo and in vitro. Literature search regarding miR-16 says that this ubiquitously expressed and highly conserved miR is one of the first few to be linked to malignancies.\textsuperscript{17,18} Importantly, miRs have been linked to all stages along the metastatic cascade in breast cancer. They can exert their action in cancers through both tumour suppression and oncogenic mechanism (as oncomirs). Currently, breast cancer can be subcategorised based on the status (+/−) of the hormone receptors, i.e. ER, PR and HER2. In our study triple negative i.e. PR-ve, ER-ve and HER2-ve were shown in the most aggressive stage IV patients.\textsuperscript{4,5,19} It is hoped that identifying breast cancer-specific miRs and their functional relevance will lead to improvements in early detection and treatment of tumours, particularly in younger patients. Identifying circulating miRs associated with distinct metastatic sites could provide another powerful diagnostic tool for clinicians to evaluate disease stage and monitor progression. Importantly, recent studies have found that miR-210, miR-328, miR-484 and miR-874 have the potential to predict prognosis or risk of recurrence.\textsuperscript{12,13,20} We have shown in our study that miR-16 may play a role in prediction of the disease in daughters of breast cancer patients.

The small sample size was a limitation of the study.

Conclusion

MiR-16 can be used as a prognostic, diagnostic as well as a predictive marker in breast cancer patients and their offspring. High expression of miR-16 in breast cancer patients suffering from stage III invasive intraductal carcinoma was noted as compared to healthy subjects. The daughter’s expression was shown to be higher than healthy individuals but lower than cases. Stage IV patients and their daughters showed an enhanced expression of miR-16 as compared to stage I.

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Conflict of Interest: None.

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