Am J Cancer Res 2015;5(8):2484-2490
www.ajcr.us /ISSN:2156-6976/ajcr0009044

Original Article

Diagnostic, prognostic and predictive value of MicroRNA-21 in breast cancer patients, their daughters and healthy individuals

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Received April 13, 2015; Accepted June 25, 2015; Epub July 15, 2015; Published August 1, 2015

Abstract: MicroRNA-21 (miR-21) located on 17q23.1 expressed in breast cancer has anti-apoptotic ability and causes tumor cell growth. It is also involved in functions such as signal transduction pathways effecting normal cell growth and differentiation. The primary objective of the study was to identify presence of miR-21 in the serum levels of stage III invasive ductal carcinoma patients and compare its expression with age matched healthy individuals and daughters of index cases. The secondary objective was to evaluate the significance of serum miR-21 gene expression with histologically proven estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) proteins. A total of 132 subjects were recruited: 50 (cases) of stage III invasive ductal carcinoma patients who had not undergone any chemotherapy or surgery were randomly picked with exclusion of females with other types of breast carcinoma. Age-matched, 50 healthy individuals (control A) were selected by purposive sampling after confirmation of no palpable lump/s in their breasts together with 32 daughters of index cases (control B). Serum tests were run on Real Time quantitative Reverse Transcription PCR, threshold cycle was determined and fold change calculated.Normality of continuous variables was assessed by Shapiro-Wilk's test, groups compared by student t-test, Mann-Whitney test and Fisher exact test, *P*-value ≤ 0.05 was considered significant. We observed that miR-21 was significantly higher in cases as compared to control A and B (P = 0.001) however control B showed significant gene expression as compared to control A (P = 0.001). The cases were also divided as positive or negative for ER, PR and HER2. High expression of miR-21 in females with stage III invasive ductal carcinoma had been calculated as compared to its age matched healthy subjects. It was observed that triple negative cases showed a greater expression of gene as compared to other groups (P = 0.001). Expression of miR-21 in daughters of the cases was significantly higher as compared to healthy controls but lesser than females with invasive intraductal carcinoma. This result strengthens the concept of inheritability of disease with prediction of miR-21 as a potentially strong diagnostic and prognostic biomarker of breast cancer.

Keywords: miR-21, breast cancer, ER, PR and HER-2

Introduction

MicroRNAs (miR, miRNA) being post transcript structures (~18-22 nucleotide) are a class of small non-coding RNAs which are regulatory in nature and have the ability to influence the translational efficiency and stability of the target messenger RNA (mRNA). These mRNA represent 5% of DNA in cell and are responsible for carrying genetic information from nuclear DNA to the cytosol where they may be used as template for protein synthesis [1].

MicroRNAs are formed by a sequence of events first in the nucleus and then in the cytoplasm. In the nucleus they are initially transcribed as primary-mi-RNA (pri-miRNA) under the action of RNase polymerase II which is ~100-1000 nucleotide in length. This is followed by the process of capping and polyadenylation. Further thispri-miRNAis cut by RNase III, DROSHA and its co-factor DGCR8 into smaller ~70 nucleotide stem loops called as pre-RNA [2, 3].

The pre-RNA travels from the nucleus to the cytoplasm by means of exportin-5. The loop region of pre-RNA is removed by DICER(RNase III) and its binding partner TRBP. A mature miRNA-miRNA* duplex is released, the single domi-

nant strand is incorporated into RISC (RNA induced silencing complex) to finally regulate gene expression by complementary-base pair interaction resulting in interference with translational ability and stability of target mRNA or it may result in its degradation. MicroRNA coding genes are present scattered in the genome in the form of single units or they may be organized into gene clusters [4, 5] Several miRs have been identified as biomarkers for various cancers; they have been analyzed in tumor tissue, plasma or serum of such patients. Till today studies show an inquisitive interest in microRNAs. This interest is being aroused for these small post transcript structures have shown an important role in tumorigenesis. Antimoleculetargeted therapy is being established against the microRNAs. TheremiRsare labeled as strong biomarkers which are under experimentation and exhibit a promising future [6, 7].

MicroRNA-21 (miR-21; has-mir-21) located on 17q23.1 has been identified in several tumors and has particularly shown high expression in breast cancer tissue especially invasive ductal carcinoma with indications of an enhanced expression with the advancement of the disease. Breast cancer is a leading cause of death in women belonging to the Asian population and miR-21 is upregulated in this disease and many other solid tumors. Researchers have pointed out that miR-21 is involved in tumor cell growth and has anti apoptotic characteristics. Relation between this miR and phosphatase and tensin homolog deleted on chromosome 10 (PTEN), tropomyosin 1 (TPMI), MCF-7 cells, maspin, metalloproteinase 3, programmed cell death 4 (PCD4) have been established by several experimentation [8-10]. An interesting recent finding regarding fenhexamid and fludioxonil which are antifungal in nature has been discovered by Teng Y et al. [11] and has been reported, these authors declare that these antifungal agents use distinct mechanism to increase miR-21 expression with downstream antiestrogenic activity. Both of these antifungal agents which have been known to be used in a number of agricultural applications inhibit estradiol induced cell proliferation and reduced cell motility [5, 11]. The level of pri-miR-21 and miR-21 are raised due to these antifungal chemicals. Their link to miR-21 may result in causing breast cancer since they increase the expression of this gene in MCF-7 breast cancer cell. This in turn reduces PDCD4 and PTEN mRNA. Links between miR-21 and p53 have been studied by Franket et al. Although carcinoembryonic antigen and CA15-3 are regarded as tumor biomarkers of breast cancer but their reliability as diagnostic markers is still under research [10, 12, 13].

Studies related to gene expression have proven that estrogen and progesterone receptors (positive/negative) are expressed as distinct disease at molecular level. Human epidermal growth factor receptor 2 (HER2) if tested positive promotes the growth of cancer cells and is normally involved in functions such as signal transduction pathways influencing cell growth and differentiation [14, 15].

A recently acceptable model of breast tumor transition says that breast cell grows from simple hyperproliferation of epithelial cells to ductal carcinoma in situ (DCIS) and then finally to infiltrating ductal carcinoma. MicroRNAs have been hypothesized to play an important role in this process. It has been reported that miR-21 up-regulation plays a parallel role in this tumorigenesis transition. Role of miRs have also been recognized in contributing to cancer initiation and progression, this is mostly done by silencing target gene expression. Over less than a decade the importance of miR found in blood serum and plasma have brought about the concept that such circulating miRs have great potential as novel non-invasive biomarker for this deadly disease, cancer [16].

Cancer chemotherapy success is impeded in multidrug resistance; this has also been associated with over expression of miR-21. It is said that highly expressed miR-21 is linked to the progression and development of multidrug resistance [17].

Although several studies have been focused on the expression of miR-21 in breast cancer tissue but little data to date is available on serum levels and no study has reported the inheritability of miRs in daughters of breast cancer patients. Therefore we aim to:

1. Identify serum miR-21 in invasive ductal carcinoma of stage III breast cancer subjects.

2. Compare the expression of serum miR-21 between breast cancer cases, aged matched healthy individuals and daughters of index cases.

3. Evaluate the significance of serum miR-21 gene expressionwith histological proven breast receptors: ER, PR and HER2 proteins and their prognostic value in breast cancer.

Methodology

A total of 132 subjects were recruited for this study, of which 50 were those suffering from breast cancer (invasive ductal) in stage III (cases), 50 subjects included age-matched healthy individuals (control A) and 32 daughters of index cases (control B). The patients were recruited from Ziauddin Cancer Hospital, North Nazimabad Karachi. The mean age of the cases was found to be 49±5.62. Controls A were recruited by purposive sampling and were disease free. Controls B were recruited if they were not suffering from breast cancer or any other cancer and did not have palpable lump/s in their breasts.

Subjects with invasive ductal carcinoma (stage III) were recruited after histological confirmation of biopsy reports. Fifty such subjects visiting the oncology clinic were randomly picked who had not undergone any chemotherapy or surgery; they were newly diagnosed patients with breast carcinoma. Females with other types of breast carcinoma were excluded. Their biopsy reports showing results of ER, PR and HER2 were noted. The daughters of these patients were given appointments to visit the cancer hospital and were invited to be a part of this study after informed consent.

5 ml blood was drawn after obtaining written informed consent from cases, controls A and B. The blood was collected in a BD gel vacutainer (yellow top), it was centrifuged at 3000 rpm. After further procedure serum was extracted and transferred to a conical bottom tube. Around 3-5 ml serum was obtained. This was stored at -80°C till further use. The PCR equipment used for the experiment was rotar gene Q, SN R-060953 Qiagen Hilden, Germany.

The first stage of the experimentation was RNA elusion in which miRNeasy serum/plasmakits were used which consisted of columns, plastic ware and reagents. Each kit was for 50 preparations; miRNeasy serum/plasma spike-in control which consisted of 10 pmol C. elegans miR-39 miRNA mimic spike-in control for serum samples was also used for this stage.

In the second stage cDNA was prepared, in this miscript RT II kits 50 was used. The contents of each kit were reagents for 50 × 20 µL cDNA synthesis reactions. The cycling conditions for real time PCR were 15 sec at 94°C for denaturation, 30 seconds at 55°C for annealing and 30 seconds at 70°C for extension. This was set at 40 cycles.

In the third stage the reaction set up for RT-PCR was initiated using miscript SYBR Green PCR kit for 1000 reactions and miscript primer assay was considered as positive control (pc) and reference for miR-21 primer. The miscript primer assay used was ce_miR-39 miscript which has binding affinity to spike-in control showing definite amplification and gene expression, this was therefore used as standard since it showed gene expression in cases and the controls. The fold change was calculated with reference to this [18, 19].

The primer for miRNA-21 designed was of sequence 5'tagcttatcagactgatgttga3'. 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. For expression analysis, this experimental design was made to compare cases with controls A and B [20].

The relative quantification of serum miR-21 in all three groups was calculated using the equation; amount of target = 2ΔΔct - ((Ct pc - Ct miR-21) - (Ct mean pc - Ct mean miR-21)). In this way the fold change for each group was calculated in order to determine the gene expression. Melting curves were generated and RT-PCR amplification performed. MicroRNA-21 were extracted from the circulation and compared between the three groups and with ER, PR and HER2.

Results

Data were entered in MS Excel 2007 and analyzed in IBM SPSS version 20 (Chicago Inc.). Frequency and percentages were used to express outcome of categorical variables. Chi-square test was used to test association among three types of patients and their categorized clinical findings. Fisher exact test was used for analyzing between cancer patients and healthy patients or with their daughters. Descriptive measures for symmetric continuous variable were expressed as mean with its standard devi-

Table 1. Comparison of gene expression

miR-21	Cases (50)	Control B (32)	*P*-value*
Mean Ct-value	28.53±2.74	33.79±7.64	0.001
Fold change	1.23±3.74	0.47±7.58	0.57

*P ≤ 0.05 is considered significant, miR-21 = microRNA-21, Ct-value = cycle threshold. Control B = daughters of cases.

ation while for skewed continuous variables median with inter-quartile range was calculated. Normality of continuous variables was assessed by Shapiro-Wilk's test. On the other hand, while comparing the same between two groups, student t-test and Mann-Whitney test was used. Mean ± SD was calculated for cycle threshold and fold changeof miR-21 in serum of cases and controls A and B. T-test was applied and *p*-value calculated between the groups. *P*-value ≤ 0.05 was considered significant.

Using Rotar gene RT-PCR, analysis of serum miR-21 in cases, controls A and B were performed. Their results were calculated and compared between the groups.High expression of miRNA-21 was calculated in serum of the cases as compared to control A whose cycle threshold was shown to be negative (P = 0.00). The gene expression in control B showed a lesser Ct value as compared to control A (P = 0.00) meaning greater gene expression. When cases and control B were compared higher gene expression was observed in the cases (**Table 1**). The individual Ct-value of each enrolled subject in both these groups is elaborated in **Figure 1**.

MicroRNA-21 was up-regulated greater than one fold change in the cases. This was calculated by applying the 2ΔΔct method (**Table 1**).

Correlation with ER in tumor tissue: The serum of those tumors with ER-ve tissue expressed mean lesser Ct value as compared to the mean Ct value of ER+ve. ER-ve showed a greater fold change of > twofold (**Table 2**).

Correlation with PR in tumor tissue: Serum miR-21 of PR-ve breast cancer tissue showed a lesser mean Ct-value as compared to PR+ve which showed greater a mean Ct-value. In addition to this PR-ve tumor tissue showed a fold change of greater then twofold (**Table 2**).

Correlation with HER2 in tumor tissue: HER2+ve cancer tissue show a lesser Ct-value with fold changegreater than onefold (**Table 1**).

Discussion

MicroRNA-21 is an emerging strong biomarker of breast cancer especially in invasive ductal carcinoma. In our study we have shown a significant expression of this gene as compared to the age matched healthy subjects. Reports from other researchers have also marked miR-21 as a potential diagnostic marker and have also shown relation to aggressiveness of the tumor. Poor follow up prognosis has been observed in patients with high miR-21 expression indicating that this gene acts as an oncogene and results in inhibiting tumor suppressor genes.

Sota et al. explained the novel use of real time quantitative reverse transcriptase polymerase chain reaction (RT-qPCR), this has proven to be efficient in miR analysis [21]. By using this method the authors noted high expression of miR-21 and also emphasized upon its diagnostic and prognostic value. In addition this study linked miR with staging of cancer. Lawrie et al. were the first to report the presence of miR-21 in B-cell lymphoma, subsequently this miR has also been postulated strongly in breast cancer and this is increasing popularity and consensus among scientists as being as non-invasive, sensitive and novel biomarker for this increasing common breast disease [22].

Laboratories have reported over expression of miR-21 in various human cancerous serum but these findings have not been unanimous, therefore analysis with bigger sample size is needed. Till to date enough evidence is available to label miR-21 as a potential biomarker for diagnostic purpose of breast cancer and this is in accordance with several researches. There is however no study indicating the expression of genes in siblings or children of the effected person. Our study attempts to link the strong inheritance capability of breast cancer with miR-21 by studying the expression of this gene in daughters of index cases. This novel finding shows that the daughters of breast cancer mothers who are suffering from stage III invasive ductal carcinoma also show some degree of miR-21 gene expression. Although the expression was lesser then that of the cases but up-regulation of the gene was analyzed in comparison with the positive control. By this we can infer that miR-21 may also be considered as a predictive marker along with its diagnostic and

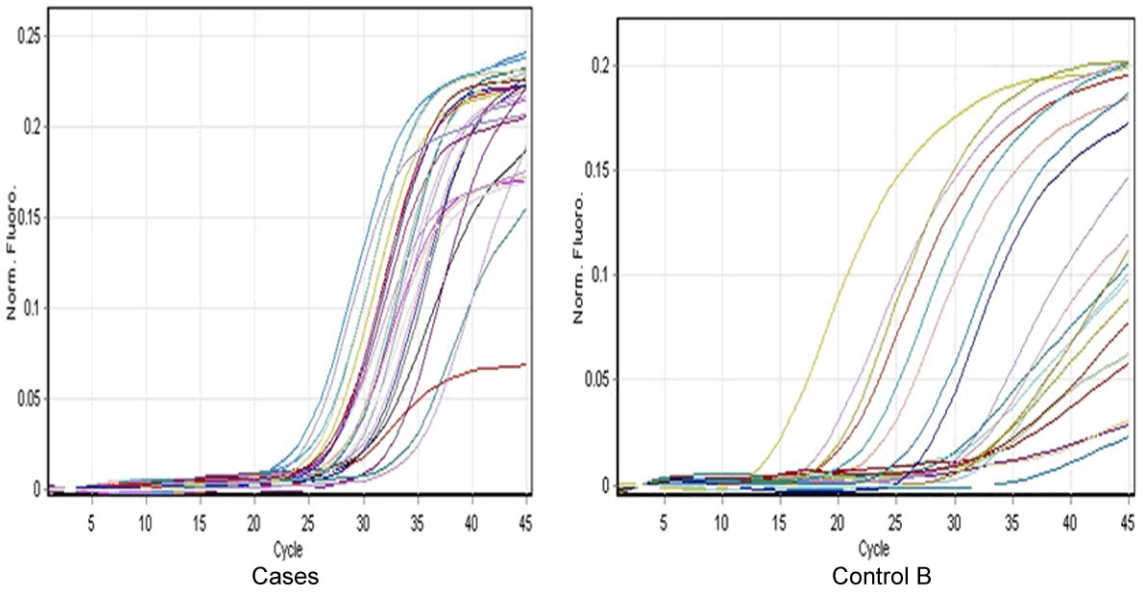

Figure 1. Comparison of cycle threshold between breast cancer patients and their daughters.

Table 2. Relation of receptors in tumor tissues with serum Ct-value and fold change

Receptors	Positive n (%)	Negative n (%)	Positive: m Ct-value	Negative: m Ct-value	Positive: FC	Negative: FC
ER	27 (54)	23 (46)	28.93±2.17	28.01±2.82	0.11±2.89	2.12±4.17
PR	29 (58)	21 (42)	29.12±2.66	27.78±2.77	0.35±2.40	2.12±4.19
HER2	29 (58)	21 (42)	28.41±2.12	28.71±3.60	0.64±3.52	1.63±3.96

n = number of cases; m = mean; Ct-value = threshold cycle; FC = fold change, ER = estrogen receptor, PR = progesterone receptor, HER2 = human epidermal growth factor receptor 2.

prognostic value. Along with miR-21 being higher in cancer tissue and serum as compared to healthy women researchers have revealed an increased level with increase in tumor size and involvement of high number of lymph nodes [16, 17, 23].

Our study showed high expression in ER and PR negative tumors and compared to their positive counter paths. Varying reports regarding relation of ER/PR status to breast cancer and miR-21 have been reported. Several researchers have shown a similar expression, Huang GI et al. has reported that ER/PR negative cancers show high expression of miR-21 and this finding is in consistence with our findings [24]. In this study this gene was found in significantly higher level in tumor tissue as compared to non-tumor tissue.

ER, PR and HER2 negative (triple negative tissue) have exhibited very high expression of miR-21 in the present study. This finding is in consistence with several other reports indicating the aggressive nature of tumor with negative receptor proteins. Metastasis of cancer is also linked to triple negative tumors and have poor prognosis and do not respond properly to chemotherapy. Correlation between clinicopathological factors and miR-21 concentration is very important especially for treatment purposes. Multi variant analysis of breast cancer in stage IV showed very high concentration of circulating miR-21 but this was independent of ER, PR and HER2. Henegham HM et alreported higher circulating levels of miR-21 in ER negative disease as compared to those individuals whose biopsy showed ER positive breast cancer [25]. These authors also tried to establish a relationship between this circulating gene, type of breast cancer (e.g. in situ or invasive), the subtype and HER2 status but no significant relation could be established.

In a study on Asian Indians and Pakistani, the receptor status in relation to age was analyzed

showing that in ages between 40-50 years an increased percentage of ER/PR negative disease was noted as compared to the younger group. However over time pathologically assessed cancers show that triple negative receptors are more common in this part of the world which may result in recurrences, resistance to treatment and metastasis in brain and spinal cord [26].

Correlational studies by Si H et al. attempted to correlate the expression of miR-21 in tissue and serum levels of same individuals, their results showed that a significant difference in gene expression was observed that is more miR-21 was expressed in tissue as compared to the serum levels [27]. Although our study showed no significant association with involvement of lymph nodes, it has been reported that patients with high miR-21 expressed confirm more lymph node metastasis. Results of formalin-fixed, paraffin embedded (FFPE) tissue samples by using qRT-PCR prove that miR-21 expression were significantly higher in patients with stage III disease of breast cancer. The authors also showed that these patients were HER2 positive. Another study on FFPE breast cancer patients showed that miR-21 was upregulated in all breast tissue being of different intraductal proliferations which included atypical ductal hyperplasia, ductal carcinoma in situ and invasive ductal carcinoma. These authors could however not link the further up-regulation with progression and aggressiveness of the tumor [2, 5, 27].

Observing the increasing and promising data of miRs there is potential to differentiate between discrete grades of breast cancer procession. The results indicate that miR-21 can be attributed to as an indicator of aggressive tumor and maybe considered as a new therapeutic target of breast cancer in the near future [27].

Conclusion

In conclusion it was determined that high expression of miR-21 in breast cancer patients suffering from stage III invasive ductal carcinoma had been calculated as compared to its age matched healthy subjects. This result can mark miR-21 as a potentially strong diagnostic and prognostic biomarker of breast cancer. miR-21 profile was also studied in daughters of the index cases. Their expression was also shown to be significantly higher as compared to the healthy individuals but lesser than the full blown disease of breast cancer. This result strengthens the concept of inheritability of this disease and this gene can also be labeled as a predictive biomarker. When Ct-values and fold-chain calculations were compared between ER, PR and HER2, higher expression was seen in negative tumor tissues which are very resistant tumors and respond poorly to chemotherapy.

Acknowledgements

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