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Identification of Circulating miRNAs as Non-Invasive Biomarkers of Triple Negative Breast Cancer in the Population of Pakistan

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ABSTRACT

Triple negative breast cancer is the heterogeneous and most aggressive known subtype of breast cancer due to absence of estrogen, progesterone and HER2 receptors. We aimed to identify a panel of circulating miRNAs which can act as diagnostic and prognostic biomarkers specifically for TNBC. The blood samples were collected from 37 TNBC diagnosed patients and 34 age-matched non-TNBC controls to investigate the expression of miRNA panel. Plasma was isolated by standard protocol of centrifugation and then isolation of total RNA including miRNA was done. About 100ng of isolated miRNA was converted to cDNA after addition of poly (A) tailing and then expression profiling was done by using real time-qPCR technique in which miR-specific DNA primers were used to increase sensitivity of reaction. Expression of miRNA panel was normalized with mir-16 and expression fold change was calculated by $2^{-\Delta\Delta Ct}$ method. Further analysis was done by SPSS, medcalc and Graphpad prism software. Area under curve analysis was used to get diagnosis outcome of selected miRNA panel. Upregulation of miRNA panel (mir-376c, mir-155, mir-17a and mir-10b) has been seen in the plasma samples of TNBC patient´s samples relative to non-TNBC control samples. The scatter plots and other statistical analysis have given differences in expression of four miRNA panel with significant p-value ($p< 0.001$), respectively. Kruskal Wallis test and Mann Whitney U test have clearly showed significance and association of miRNA panel with clinicopathological characteristics of breast cancer which have revealed their role as diagnostic biomarkers. AUC curve analysis also remarks their high sensitivity and specificity. We have identified panel of four miRNAs which can act as circulating diagnostic and prognostic biomarkers in triple negative breast cancer in the population of Pakistan. Especially, mir-376c, mir-155 and mir-17a may act as early stage biomarkers in TNBC and mir-10b is found to be late stage biomarker which can also act as prognostic biomarker in TNBC.

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MWA and S Sadaf designed the experiment, supervised the research and reviewed the manuscript. JS and S Shahzadi collected the clinical samples. Samiah Shahid and JS performed the experiments. JS prepared the manuscript.

Key words
Breast cancer, microRNA, Real-time PCR, Diagnostic biomarker, Circulating.

INTRODUCTION

Breast cancer is the malignant disease which affects breast tissue; its mortality rate is very high amongst women worldwide especially in Asian countries. In Asia, Pakistan has highest rate of prevalence of breast cancer *i.e.,* for every 9 women, 1 is diagnosed with breast cancer (Menhas and Umer, 2015). About 1 million cases of breast cancer have been detected annually worldwide and it is a major cause of death in women (Maryam *et al*., 2018). There are several factors which may lead towards breast cancer (Harirchi *et al*., 2010), such as age, early menstruation, absence of menstrual periods, genetic disorder, lack of breastfeeding, less physical activity and alcoholic consumption (DeRoo *et al*., 2010; Ghiasvand *et al*., 2011).

Triple negative breast cancer (TNBC) is heterogeneous and one of the aggressive subtypes of breast cancer (Sãrlie *et al*., 2001; Almeer *et al*., 2018). Its prognosis outcome is mostly poor and chances of relapse are greater than other subtypes because of the absence of estrogen receptors, progesterone receptors and human epidermal receptor 2 whose presence helps in treatment of disease by direct targeting these receptors (Chang *et al*., 2011).

Despite of available diagnostic and prognostic methods, there is still frequent need of sensitive, specific and non-invasive method of screening, in order to cure and better treatment of this type of aggressive cancer (Di Leva and Croce, 2010). New research approaches have demonstrated that miRNAs which are a class of regulators of 50% genes and are related to certain diseases may serve as biomarkers (Fish *et al*., 2008; Wang *et al*., 2008). MicroRNAs are short non-coding RNA molecules, consists of 22-24 nucleotides, translating any protein sequence, while playing crucial roles in the

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gene regulation. Since the discovery of lin-4 miRNAs in Caenorhabditis nematode, lot of research has been done on different disease relating miRNAs (Lee *et al*., 1993). They play crucial role in controlling apoptosis, differentiation, cell growth and development, metabolism and cell proliferation inside cells (Kim *et al*., 2009). As miRNAs are known to be present in specific manner in tissues and also secreted in blood as free circulating miRNAs so they act as diagnostic and prognostic biomarkers for breast cancer (Takahashi *et al*., 2015). In 2005, Iorio *et al*. (2005) have first time conducted study on breast cancer tissue and its associated miRNAs. The presence of mir-21as first oncomir in breast cancer was also found by same group (D'Ippolito and Iorio, 2013). After that mir-155 and mir-10b were most studied miRNAs related to breast cancer. In 2014, Sahlberg *et al*. (2014) identified mir-18b, mir-103, mir-107 and mir-652 as prognostic biomarkers for TNBC. Similarly, Liu *et al*. (2015) studied mir-374b-5p, mir-27b-3p, mir-126-3p and mir-218-5p expression in tumour samples of TNBC patients and confirmed their relevance with TNBC.

In our study, we have selected four miRNAs on the basis of their strong association with breast cancer and their important role in the regulation of disease specific genes. Their deregulated expression levels in plasma of TNBC versus non-TNBC have corroborated that they act as non-invasive early stage diagnostic biomarkers but also as a prognostic markers.

MATERIALS AND METHODS

Sample collection and processing

Blood samples of diagnosed 37 TNBC and 34 non-TNBC were collected from Jinnah and INMOL Cancer Research Hospital, Lahore and informed consent was taken from patients as well as from healthy volunteers. From Ethical Review Board of the School of Biological Sciences [Ref. No. 873/12] conducted study was approved. Clinicopathological parameters of the patients were obtained from tumor registry record available from respective hospital. Blood samples which were taken in 5cc EDTA collection tubes processed as per approved protocol in order to separate plasma. Briefly, spinning of blood at 2,500 x g for 5 min in refrigerated centrifuge then transferring of plasma in new eppendorf tube for second high speed spinning was done at 4,500 x g for 5 min to get cell free clear plasma. Aliquots of 500 µl plasma were formed and stored at -80°C.

Primer designing

For quantification of selected miRNAs, DNA primers were used, which were designed according to protocol described by Balcells *et al*. (2011) (Table I).

Table I.- mir-specific DNA primers used in miRNA analysis.

Oligo name	Sequence (5'→3')
mir-16	F: CGC AGT AGC AGC ACG TAA ATA T
	R: GGT CCA GTT TTT TTT TTT TTT TCG CCA
mir-10b	F: CAG TAC CCT GTA GAA CCG AAT T
	R: CAG GTC CAG TTT TTT TTT TTT TTT CAC A
mir-376c	F: CGC AGG GTG GAT ATT CCT TCT A
	R: CAG GTC CAG TTT TTT TTT TTT TTT AAC A
mir-155	F: CGC AGC TCC TAC ATA TTA GCA T
	R: CAG GTC CAG TTT TTT TTT TTT TTT GTT A
mir-17a	F: AG CAA AGT GCT TAC AGT GCAG
	R: GGT CCA GTT TTT TTT TTT TTT TCT AC

MicroRNA isolation and cDNA synthesis

From 300 µl plasma aliquots, total RNA was insulated by using NucleoSpin® miRNA Plasma kit (MACHEREY-NAGEL Co., Germany) as per defined protocol and then quantity of miRNA isolated from plasma was measured on NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific™ (Pvt.) Ltd., USA) with RNase free water used as blank. About 100 ng of reconstituted miRNA was then used for poly A tailing (-A_{15}) reaction by using poly (A) polymerase Cat. # AM2030 (Invitrogen™ by Thermo Fisher Scientific™ (Pvt.) Ltd., USA). A 20 µl reaction was set up by combining 4 µl of 5x PAP buffer, 2.5 mM $MnCl_2$, 1 mM ATP and 1 unit of poly-A polymerase in an RNase-free PCR tube. The mixture was incubated at 37°C for 60 min and the enzymatic reaction was inactivated by heating at 65°C for 20 min. The mixture was next processed for the cDNA synthesis using M-MuLV RevertAid Reverse Transcriptase enzyme (Thermo Scientific (Pvt.) Ltd., USA) according to the recommended protocol. Briefly, 8 µl of poly (A) polymerized RNA was mixed with 1 µM of universal RT-primer (5′-CAGGTCCAGTTTTTTTTTTTTTTGT-3′), incubated at 65°C for 5 min and chilled rapidly on ice. 1 x RT buffer, dNTPs mix (10 mM) and M-MuLV RevertAid Reverse Transcriptase enzyme (200 U) were added thereafter and the reaction (final volume 20 µl) was preceded with incubation at 42°C for 1 h. To stop the reaction, the mixture was heated at 70°C for 10 min.

Real-time qRT-PCR analysis

For quantitative PCR analysis, Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific (Pvt.) Ltd., USA) was used. The qPCR reactions were set up in sterile 0.2 ml sterile tubes by mixing 1 µl of 10x

diluted cDNA with 250 nM of each primer set and 5 µl of 2X Maxima SYBR Green/ROX qPCR Master Mix fluorescein, in a total volume of 10 µl. Cyclic conditions set on BioRad CFX96™ Real-Time System were: 95°C for 10 min followed by 40 cycles of heating at 95°C for 15 sec and annealing at primer specific temperatures between 55-60°C for 30 sec with extension at 72°C for 30 sec. Melt curve analysis (65°C to 95°C) were performed at the end of each thermal profile to check the specificity of amplification reactions. All Q-PCR experiments were performed in replicates and the quantification is based on the estimation of threshold cycle (ΔC_t value). Raw data was normalized by subtracting the Ct value of normalizer mir-16 from the test prior to analysis. No template control is used as a negative control and mixture of 71 samples (1µl from each sample) was taken as a positive control. The formula $2^{-\Delta\Delta Ct}$ was used to compare the relative fold change of miRNA in TNBC to that of non-TNBC (Livak and Schmittgen, 2001).

Statistical analysis

Clinical data is presented as mean ± SEM. A student's T-test (for two groups) or one-way ANOVA (for three or more groups), Mann-Whitney U test and the significance of differences between groups was estimated by the Kruskal–Wallis test for *k*-group comparisons while Pearson correlation is done to see correlation between selected miRNA panel by using SPSS version 21.0 (IBM Corp, Armonk, NY, USA). Receiver operating characteristic (ROC) analysis was performed by using MedCalc for Windows, version 18.0 (MedCalc Software, Ostend, Belgium) Receiver-operating characteristic (ROC) curves were generated to assess the diagnostic accuracy of each miRNA and the sensitivity and specificity of the optimum cutoff point were defined as those values that maximized the area under the ROC curve (AUC). Scatter plots were made by using GraphPad Prism 5 (GraphPad, La Jolla, California) in order to define fold change expression of target miRNAs. The results were considered to be statistically significant, when $p < 0.05$.

RESULTS

Selection of reference gene

The selection of reference gene is very important for data analysis of miRNA data obtained from different samples, so from previous studies two reference genes mir-16 and mir-484 were selected (Liang *et al*., 2007; Davoren *et al*., 2008; Hu *et al*., 2012) and applied on our test data set (data not shown). Only mir-16 has shown stable expression throughout all samples and has been chosen for final analysis of miRNAs expression.

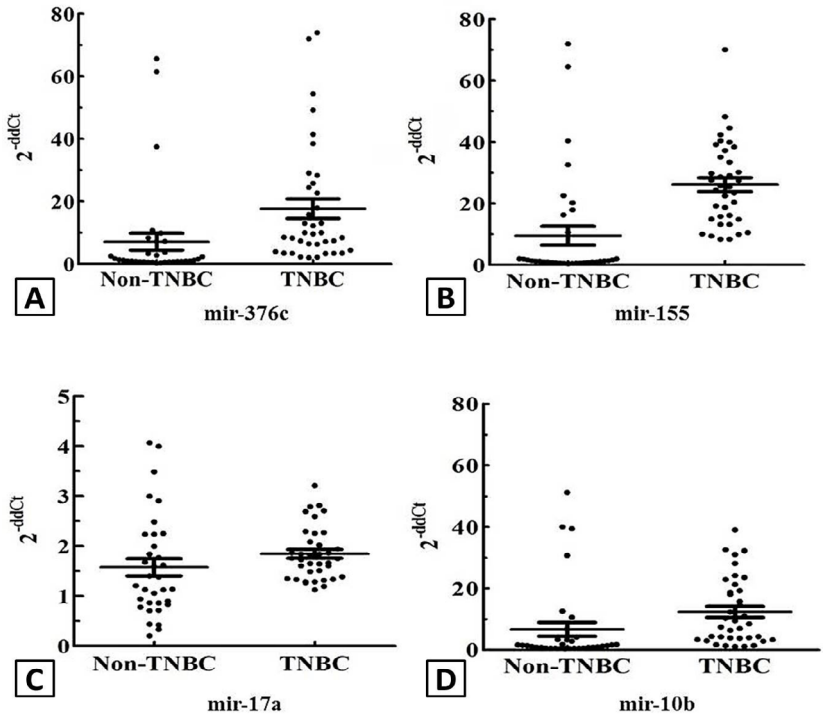

Fig. 1. Scatter plots are representing high expression fold change in mir-376c, mir-155, mir-17a and mir-10b with significant p-value ($p < 0.05$).

Up-regulation of miRNA panel in triple negative breast cancer samples

The expression levels of mir-376c, mir-155, mir-17a and mir-10b were checked by using qRT-PCR reaction, respectively, these miRNAs were known to be significant contributors in the pathogenesis of breast cancer. All four miRNAs were found to be upregulated in TNBC relative to non-TNBC plasma samples. The Ct values obtained by expression profiling of miRNA panel were normalized by using endogenous mir-16 which was used as a reference gene. Mean expression fold change (FC) of mir-376c, mir-155, mir17a and mir-10b observed was 17.38, 26.21, 1.84 and 12.65, respectively in TNBC plasma samples while expression FC was 6.53, 9.57, 1.02 and 6.72, respectively in non-TNBC plasma samples (Fig. 1). p-values were calculated by Mann-Whitney U test ($p< 0.0001$).

Clinicopathological association with mir-376c, mir-155, mir-17a and mir-10b

Association of miRNA panel with clinicopathological characteristics of TNBC patients was calculated by Independent samples Mann-Whitney U test and Independent samples Kruskal Wallis test. The expression fold change of miRNAs with respect to these individual features was categorized by taking mean and standard error (Table II). Standard error is used to measure the accuracy, with which a sample signifies a population. Independent samples Mann Whitney U test and Independent samples Kruskal Wallis tests were used to see significance of clinicopathological characteristics with disease. Overall significance of age group, stage and grade with mir-376c, mir-155 and mir-17a was found to be $p< 0.005$. Clinical types of collected diseased plasma samples didn't showed statistical significance but T, N and M have given significant expression levels with mir-376c and mir-155, respectively ($p< 0.05$) as shown in Table II. The mean expression fold change of mir-376c and mir-155 was found to be increased in stage II (44.1 and 39.4, respectively) and then relative decrease of their expression was observed in stage III (12.3 and 28.4) and IV (5.1and 11.6), respectively. Opposite expression fold change was observed in mir-10b with decreased expression at stage II (7.9) and then subsequent increase at stage III (12.3) and IV (16.9) occurred. But mir-17a showed increased expression at stage II with constant expression fold change at stage III and stage IV, respectively (Table I; Fig. 2A).

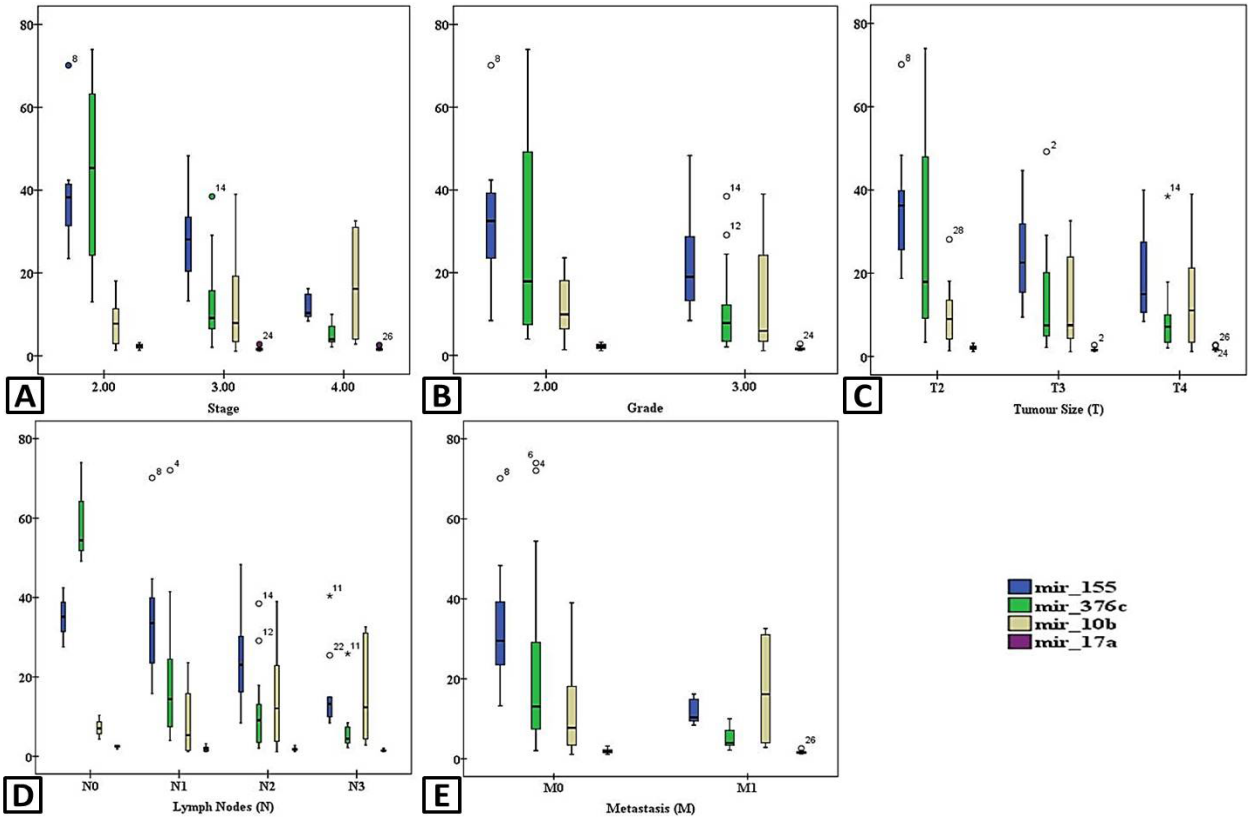

Fig. 2. Box and Whisker plots are showing stage, grade, tumour size, lymph nodes involvement and metastasis association with mir-155, mir-376c, mir-10b and mir-17a, respectively in TNBC patient's plasma samples.

Table II.- Clinicopathological characteristics association with miRNA panel.

Parameters	TNBC (n=37)	mir-376c	mir-155	mir-17a	mir-10b
Age					
25-35	11	5.9±1.2	21.2±3.6	1.6±0.0	13.2±3.3
36-45	09	15.6±4.9	26.2±3.4	1.6±0.1	11.9±3.4
46-55	12	31.4±7.1	24.3±3.1	2.2±0.1	13.1±3.3
56-65	04	10.7±3.73	44.5±14.8	2.2±0.1	10.3±8.8
66-75	01	10.1±0.0	48.3±0.0	1.1±0.0	14.6±0.0
p-value		0.016	0.03	0.020	0.910
KW Test		12.215**	6.474**	11.691**	0.999^{ns}
Type					
Infiltrating Ductal Carcinoma	02	9.15±0.8	37.1±11.2	1.4±0.2	9.3±5.3
Invasive Ductal Carcinoma	34	17.5±3.4	25.4±2.4	1.8±0.1	12.5±1.9
Lobular Carcinoma	01	29.1±0.0	30.2±0.0	1.7±0.0	24.1±0.0
p-value		0.541	0.400	0.562	0.472
KW Test		1.228^{ns}	1.833 ^{ns}	1.153^{ns}	1.499^{ns}
Stage					
2	08	44.1±7.9	39.4 ± 4.9	2.3 ±0.2	7.9±2.1
3	19	12.3±2.2	28.4± 2.2	1.7±0.1	12.3±2.6
4	10	5.1 ± 0.8	11.6 ± 0.9	1.7±0.1	16.9±3.9
p-value		0.000	0.000	0.018	0.336
KW Test		19.31***	22.05***	8.09**	2.182^{ns}
Grade					
G2	14	28.5±6.7	32.8 ± 3.9	2.1 ±0.1	11.5 ±1.9
G3	23	10.2±2.0	21.9 ± 2.5	1.7±0.1	13.4±2.6
p-value		0.009	0.025	0.021	0.871
MW U Test		73**	85**	83**	149^{ns}
Tumour size (T)					
T2	12	28.8±7.3	54.8±3.9	2.1±0.1	9.9±2.2
T3	12	14.3±4.3	24.6±3.7	1.6±0.1	14.3±3.6
T4	13	9.4±2.7	18.6±2.7	1.8±0.1	13.7±3.4
p-value		0.03	0.009	0.079	0.812
KW Test		6.99**	9.39**	5.066^{ns}	0.415^{ns}
Lymph nodes (N)					
N0	03	59.1±7.5	35.1±4.2	2.4±0.2	7.2±1.7
N1	10	21.9±6.5	35.1±4.8	1.9±0.2	8.24±2.5
N2	14	11.7±2.8	24.2±3.0	1.8±0.2	14.2±3.1
N3	09	7.1±2.5	16.3±3.5	1.5±0.1	16.8±4.2
p-value		0.003	0.009	0.09	0.297
KW Test		13.7**	11.4**	6.40^{ns}	3.68^{ns}
Metastasis (M)					
M0	27	22.1±4.1	31.8±2.3	1.9±0.1	11.1±1.9
M1	10	5.1±0.8	11.6±0.9	1.6±0.1	16.9±3.9
p-value		0.001	0.000	0.230	0.180
MW U Test		37***	4***	96^{ns}	168^{ns}

The table is representing mean values and standard error of means calculated by normalized expression fold change of four miRNA panel. p-values and scores of MW U test and KW test are also given. Abbreviations and notations are given to show which miRNA expression is giving statistical significance or which is not *i.e.,* KW Test= Independent samples Kruskal Wallis, MW U Test=Independent samples Mann-Whitney U test, *P*-value < 0.05 = *, *P*-value < 0.01 = **, *P*-value < 0.001 = ***, *P*-value > 0.05 = ns (non-significant) and G= grade.

Similarly, with grade II high expression fold change was observed in mir-376c, mir-155 and mir-17a and opposite trend was seen in mir-10b with high expression fold change at grade III (Table II; Fig. 2B).

In case of tumour size, involvement of lymph nodes and metastasis, mir-376c, mir-155 and mir-17a expression fold change was found to be kept decreasing with advanced stages *i.e.,* T3-T4, N2-N3 and M1 thus confirming their role in early diagnosis of TNBC patients while mir-10b showed its involvement in late stage and advanced metastasis, so it acts as prognostic marker as well (Table II; Fig. 2C-E).

Diagnostic potential of mir-376c, mir-155, mir-17a and mir-10b in plasma

For the selection and validation of selected miRNA panel as biomarkers for TNBC, sensitivity and specificity was checked in the target population samples. Receiver operating characteristic curve, *i.e.* ROC curve, was used to clarify the diagnostic ability of a binary classifier system as its discrimination threshold is varied. ROC curve is an example of AUC (Area under curve) which is used to classify the two groups and helps to predict diagnostic value of target miRNA panel (Fig. 3). ROC has given sensitivity *i.e.,* 91.83, 100, 94.59, 81.08 and specificity *i.e.,* 73.53, 73.53, 50, 73.53 for mir-376c, mir-155, mir-17a and mir-10b, respectively, calculated by J-youden index while AUC calculated were 0.866, 0.847, 0.657and 0.773, respectively. Significance calculated by Mann-Whitney test was $p< 0.0001$ for each miRNA (Fig. 3). The comparison of ROC between four selected miRNAs showed mean AUC was 0.785.

Correlation between clinical parameters and miRNA panel in TNBC plasma samples

In order to confirm the correlation between clinical parameters and miRNA panel and to use these miRNAs together as diagnostic panel, Pearson Correlation was calculated (Table III). Clinical parameters have given statistically significant correlation with miRNA panel but mir-155 has showed correlation with mir-376c and mir-10b, respectively, while mir-376c showed strong correlation with mir-155 and mir-17a. In case of mir-17a correlation has been seen only with mir-376c and mir-10b was observed to have correlation only with mir-155. By observing overall data and Pearson correlation, we can suggest that these miRNAs can act as diagnostic panel for TNBC plasma samples.

Table III.- Correlation of clinical parameters with miRNA panel and between miRNA panel.

Variables		Correlations			
		mir-155	**mir-376c**	**mir-10b**	**mir-17a**
Stage	Pearson Correlation	-0.729**	-0.700**	0.298	-0.436**
	p-value	0.000	0.000	0.078	0.008
Grade	Pearson Correlation	-0.390*	-0.470**	0.089	-0.429**
	p-value	0.019	0.004	0.604	0.009
T	Pearson Correlation	-0.525**	-0.424**	0.144	-0.238
	p-value	0.001	0.010	0.402	0.161
N	Pearson Correlation	-0.520**	-0.629**	0.329*	-0.433**
	p-value	0.001	0.000	0.050	0.008
M	Pearson Correlation	-0.664**	-0.402*	0.249	-0.210
	p-value	0.000	0.015	0.144	0.219
mir-155	Pearson Correlation	1.00	0.389*	-0.369*	0.268
	p-value		0.019	0.027	0.114
mir-376c	Pearson Correlation	0.389*	1	-0.188	0.554**
	p-value	0.019		0.271	0.000
mir-10b	Pearson Correlation	-0.369*	-0.188	1	-0.074
	p-value	0.027	0.271		0.667
mir-17a	Pearson Correlation	0.268	0.554**	-0.074	1
	p-value	0.114	0.000	0.667	

Pearson correlation is calculated between miRNA panels and also between clinical parameters and miRNA panel. **, Correlation is significant at the 0.01 level (2-tailed). *, Correlation is significant at the 0.05 level (2-tailed).

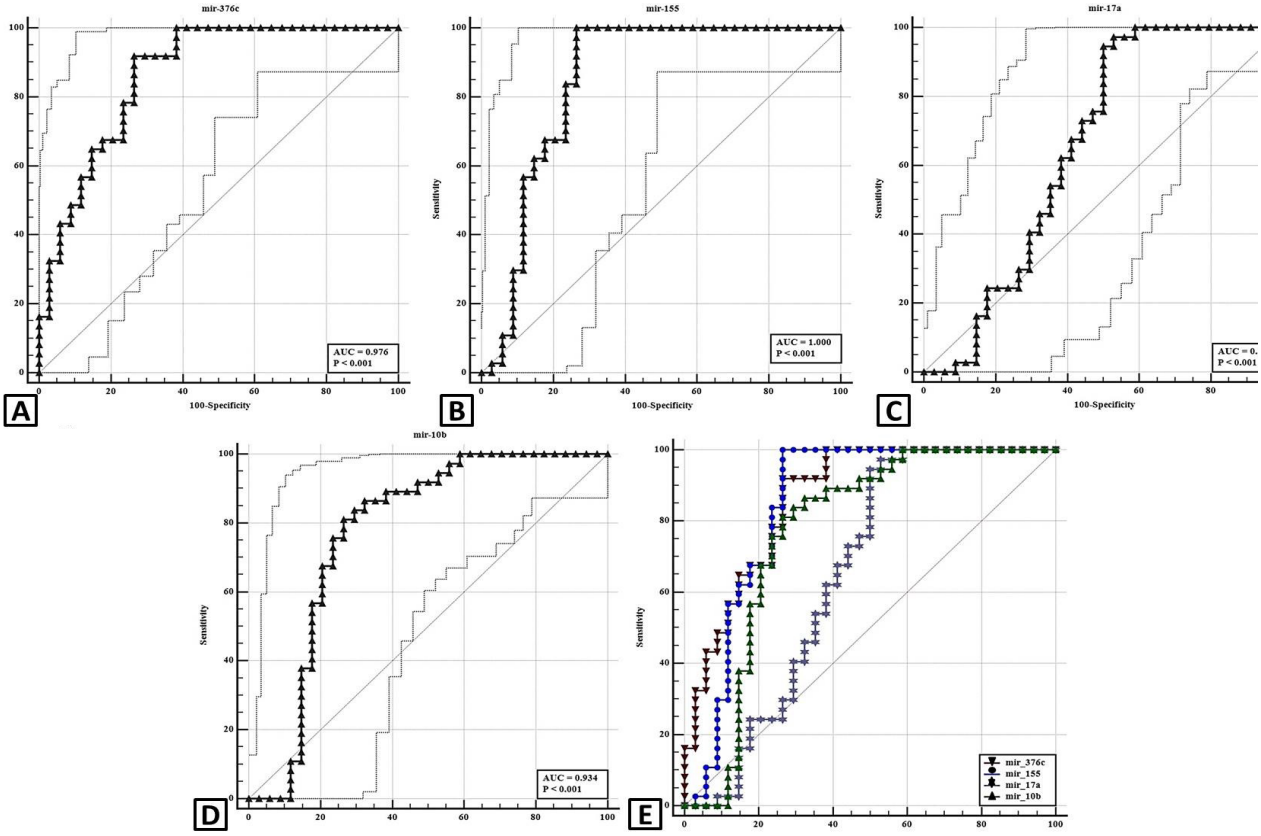

Fig. 3. Receiving Operating Curves (ROC) representing AUC, sensitivity and specificity of mir-376c, mir-155, mir-17a and mir-10b and comparison of miRNA panel for the assessment of biomarkers diagnostic outcome in TNBC vs non-TNBC.

DISCUSSION

In present study, it was assumed that the selected miRNAs would show high expression in disease state as they were pronounced as 'oncomirs' in previous studies and would give relevance with clinicopathological features. TNBC is the heterogenous malignancy and very hard to cure due to absence of ER, PgR and Her2 receptors on diseased cell surface which hinder the targeted treatment (Blenkiron *et al*., 2007). Previous studies have demonstrated that mir-155 is upregulated in breast cancer (O'Day and Lal, 2010; Iorio *et al*., 2005; Sun *et al*., 2012). In another study, which is contrary to our study results, demonstrated that mir-155 expression in breast tumour has been considerably related with high neoplasm grades, lymph node metastasis and advanced cancer stage (Cortez *et al*., 2012). BRCA 1 is the outstanding finding associated to the role of miRNA-155 in breast tumor. It has a role in DNA repair and cell cycle procession. Anomalies of BRCAI are associated to raise danger of breast tumor. In a current study, by the over expression of mir-155 in BRCA1 wild-type cells demonstrated a same phenotype to mutates, showing that Breast cancer1 (BRCA1) perform functions by controlling mir-155 (Chang *et al*., 2011).

As TNBC is prominent in familial breast cancer having involvement of BRCA genes so mir-155 expression profiling gives the best biomarker for triple negative breast cancer. In present study, it showed high expression in early stage and grade so we can suggest mir-155 as early diagnostic marker for TNBC plasma samples (Table II; Fig. 2).

Similarly, mir-376c one of the highly unregulated miRNA in our panel was described to be unregulated in plasma of breast cancer (Cuk *et al*., 2013) have given consistent results in TNBC plasma samples with high significance with clininicopathological features. It has given high expression in early stage and grade without involvement in M1 and late stage lymph node metastatsis so it would be our early predictive diagnostic biomarker for triple negative breast cancer (Table II; Fig. 2). High expression of mir-376c in plasma of TNBC is only studied by us.

Many studies have demonstrated that mir-10b has a role in metastasis and gives high expression in metastatic

cell lines and tumour tissues, while other studies showed its low expression in non-metastatic tissues and cell lines (Ma *et al*., 2007; Radojicic *et al*., 2011; Tang *et al*., 2012). Current study showed consistent results with previous studies and has given high expression fold change in late stage and advanced metastasis and high involvement in of distant lymph nodes (Table II; Fig. 2). In case of mir-17a, expression levels are relatively higher throughout all stages without much difference but didn't observed to be involved in late stage metastasis (Table II; Fig. 2) which is the contradictory observation with respect to previous studies which declared its role in aggressive behavior of breast cancer and could induce metastasis partially by targeting the extracellular matrix (ECM) proteins TIMP2 and TIMP3 (Yang *et al*., 2015).

Diagnostic value of miRNA panel has been calculated individually (0.866, 0.847, 0.657and 0.773) and in comparison mean AUC was 0.785, which describes that mir-376c, mir-155, mir-17a and mir-10b can be used in panel as a diagnostic as well as prognostic markers in triple negative breast cancer to increase the specificity and sensitivity (Fig. 3). While Pearson correlation between miRNAs also supported the current hypothesis of our conducted study (Table III).

CONCLUSION

It is concluded that plasma-based high expression fold change of mir-376c, mir-155, mir-17a in early stage and grade would consistently be engaged for diagnosis and prognosis of triple negative breast cancer and give non-invasive procedure to diagnose TNBC at early stage. However, mir-10b acts as late stage diagnostic and prognostic plasma TNBC biomarker. Further validation is required by taking high sample number and comparison with other subtypes of breast cancer.

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Statement of conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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