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RESEARCH ARTICLE

Circulating Micro-RNAs as Non-invasive Molecular Biomarkers for Early Detection Breast cancer.

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Abstract

In recent years, circulating miRNAs have attracted a great deal of attention as promising novel markers for various diseases. We investigated their potential to serve as minimally invasive, early detection markers in early stages breast cancer in blood plasma and non-cancerous familial high risk individuals. We profiled miRNAs extracted from the plasma of early stages breast cancer patients ,stages one and two (taken at the time-point of diagnosis) and 23 familial high risk individuals as well as 39 healthy control individuals using syber green RT-qPCR. MiRNA 21 was shown to be significantly up regulated with p-value 0.0001 ,while miR-let 7c shown to be significantly down regulated in the plasma of early stages breast cancer patients with p-value 0.0160. On the other hand, both of miR-21 and miR-148b had significant differences between early stages breast cancer patients and familial non-cancerous high risk individuals. In conclusion, the identified miRNAs might be of potential use in the development of a multi markers blood-based test to complement and improve early detection of breast cancer and to test the hypothesis that miRNAs profiles reflect the risk of breast cancer in women at high risk due to strong family history. Such multimarkers blood test might for instance provide a prescreening tool, especially for younger women, to facilitate decisions about which individuals to recommend for further diagnostic tests.

Introduction:-

The potential of circulating microRNAs as biomarkers for cancer early detection is particularly relevant to breast cancer because breast cancer(BC) is the most frequent carcinoma and second most common cause of cancer-related mortality in women (Bombonati & Sgroi 2011), with an estimated 1.67 million new cancer cases diagnosed worldwide during 2012 which represents 25% of all cancers (Ferlay et al. 2014) . In Egypt, breast cancer is estimated to be the most common cancer among females accounting for 33.8%, 26.8% and 38.7% in Lower, Middle, and Upper Egypt respectively. By 2050, the incidence of breast cancer will increase 3-folds relative to 2013 (Ibrahim *et al.*, 2014). Its heterogeneous character is reflected in the classification into four intrinsic subtypes (luminal A, luminal B, basal-like, and +ve HER-neu). Mortality rates have continued to decrease over the years due to the advanced tools in early diagnosis and treatment. (Jemal et al.,2011).

One of the major breast cancer risk factors is the family history of the disease. Women with positive BC family history were three times more likely to get the disease. It was reported that women with one, two, three or more first-degree affected relatives have an increased breast cancer risk when compared with women who do not have an affected relative (risk ratios 1.8, 2.9 and 3.9, respectively). (Collaborative Group on Hormonal Factors in Breast Cancer .,2001:Elkum et al. ,2014)

Early cancer detection remains a major challenge in breast cancer research as it holds promises to result in a more favorable disease outcome. However, the current standard screening method, mammography, uses potentially harmful ionizing radiation and has false positive rates up to 20% (Blanchard et al. 2006). The diagnosis of breast cancer relies on the histological examination of tissue biopsies, or cytology of fine-needle aspirates, which are both invasive procedures. Known serum-based tumor markers, such as CA15.3 and CEA, cannot be used for breast cancer detection due to low sensitivity.

MicroRNAs (miRNAs) are a group of small non-coding RNAs able to regulate gene expression at the post transcriptional level by binding to the 3 untranslated region (UTR) of target mRNAs. Measuring circulating miRNAs in the plasma could offer an important complement of existing diagnostic tools allowing improved performance in breast cancer screening and detection (Zhu et al., 2009) as it had important advantages include the possibility of their repeated measurement in a noninvasive manner as well as their remarkable stability in plasma/serum, where they circulate even outside of exosomes and are stable due to their binding to Argonaute proteins. (Mitchell et al., 2008: Turchinovich et al., 2011: Arroyo et al. 2011)

Aim of the work:-

There is a need for developing novel markers that are minimally invasive, for the improved detection of breast cancer Therefore, the development of a blood based diagnostic tool would be a significant advantage as it would permit screening to begin at a younger age and could potentially successfully diagnose tumors which are not detectable by mammography and to test the profiles that reflect the risk of breast cancer in women at high risk due to strong family history.

Patients selections:-

This study was approved by the Ethical Committee of the National cancer institute (MS2001415010.4/2014), Egypt. Blood samples were collected from 20 early stages breast cancer patients and 23 individuals with strong family history (first relatives) who hadn't any disease history except breast cancer, as well as 39 healthy female volunteers who served as controls. Patient's blood samples were collected in 2013 and 2014 at the time-point of diagnosis before they underwent any therapeutic procedures, such as surgery, radiation or systemic therapy.

For, histopathological characteristics and tumor stage were assessed based on histobiopsy results and imaging techniques. Control blood samples were collected from healthy women with no history of malignant diseases, and no current inflammatory condition. The median ages of women with BC , familial high risk individuals (FHR) and control group were 44.5, 36 and 32 years, respectively.

Blood processing and miRNA isolation from plasma:-

EDTA blood samples were collected from cases and control individuals and processed for plasma within 2 hrs. of collection. Blood was centrifuged at 1,300 g for 20 min at 15 ºC. The plasma was transferred into microcentrifuge tubes followed by a second high-speed centrifugation at 14,000 g for 10 min at 15 ºC to remove cell debris and fragments. The plasma was aliquoted into cryo vials, snap-frozen in liquid nitrogen and stored at -80 ºC until use. Total RNA (including miRNAs) was extracted from 250 **µ**L of plasma. Denaturation and phase separation were conducted according to manufacturer's protocol, miRNeasy Mini Kit, Catalog no 217004. (Qiagen, Germany**)** with a minor modification: After washing miRNAs were eluted in 20 µL of RNase-free water (Cuk et al. ,2013).

cDNA syntheses for total RNA:-

The miscripte RNA reverse transcription kit miScript II RT Kit catalog no.218161 (Qiagen ,Germany) was used for cDNA syntheses from total RNA and miRNA. The reverse transcription master mix was prepared on ice as following (µl 5x miscript RT, 1 µl 10x miScript Nucleics Mix , 2 µl 5x miScript HiSpec Buffer) . The reverse transcription master mix contains all components required for first-strand cDNA synthesis except template RNA. 6 µl template RNA were added to each tube containing reverse transcription master mix, The samples were Incubated for 60 min at 37ºC then Incubated for 5 min at 95ºC to inactivate miScript Reverse Transcriptase Mix and stored at -20 ºC till the PCR step (Iorio et al., 2005).

MiRNAs Primers used are:-

MiR-21, miR-155, miR-409-3p, miR-127-3p, miR-202, miR-145b, miR-148b, miR-10b, miR-652 and miR-let7c all these primers were obtained from miRNA-Specific miScript Primer Assay (Qiagen ,Germany).

Real- Time Polymerase Chain Reaction Technique:-

Using miScript SYBR Green PCR Kit, catalog no.218073(Qiagen,Germany) Real-time PCR was carried out with an Applied Biosystems (viiA7) thermocycler under the following conditions (10 min.at 95 ºC, the denaturation at 95 ºC for 15 sec., annealing at 55 ºC for 1 min. repeating for 40 cycles (Zhu et al., 2009).

The relative gene expression of the target miRNAs was analyzed and calculated using Real –time Quantitative PCR and the Livaks $2^{-\Delta\Delta Ct}$. Equation (Livak & Schmittgen .,2001)in relation to miR-16 as internal control genes for plasma samples.

-Fold change is calculated by the equation $2^{-\Delta\Delta Ct}$.
-Fold regulation is calculated by (-1/fold change).
-Fold regulation for control is equal zero.
- (-) Fold regulation indicates down regulation.
- (+) Fold regulation indicates up regulation.

All statistical analyses were performed using normalized data. Raw data were normalized to miR-16 .Wilcoxon rank sum tests with continuity correction were used to identify miRNAs that were differentially expressed between cases and controls in the study. To detect correlations between miRNA expression levels and clinicopathological (breast cancer cases), the following nonparametric tests were used: Wilcoxon rank sum test (for relating miRNA expression to binary categorical variables), a two-tailed $p \leq 0.05$ was considered statistically significant (Cuk et al. ,2013) .

Receiver operating characteristic (ROC) curves constructed and areas under the curves (AUC) calculated as well as specificities for fixed sensitivity values with corresponding 95% confidence intervals (CI).

Results:-

The present results showed up regulation of 4 out of 10 studied miRNAs while 5/10 miRNAs showed down regulation and only one had no change as shown in table(1). In accordance to familial high risk group 5 out of 10 studied miRNAs were shown to be highly significant down regulated as shown in table(2). In comparison analysis between early stages BC versus familial high risk group both miR-21 and miR-148b as shown in table(3) had significant changes between the two groups. Also, the results showed that miR-21, miR-let 7c, miR-155 , 202, 10b ,148b,145b,and miR-409-3P are significantly correlated with distinct clinicopathological characteristics.

(Table 1): The mean fold regulation of different miRNAs in early stages breast cancer patients.

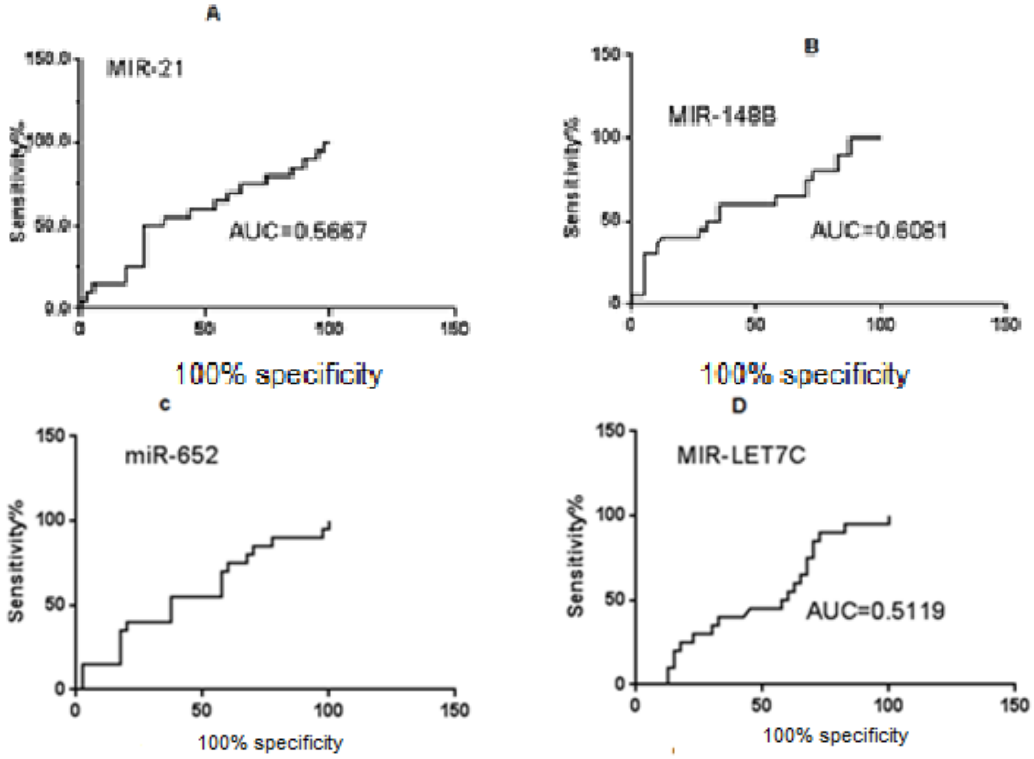
MiRNAs	Mean of fold regulation	Standard error	P-VALUE	Regulation
MiR-21	30.53	16.07	**< 0.0001*** **	Up regulation
MiR-LET 7C	-35.63	14.47	**0.0160** **	Down regulation
MiR-145B	-1.620	9.655	0.4684	No change
MiR-148B	11.21	8.477	0.1466	UP regulation
MiR-10B	24.81	25.96	0.9	Up regulation
MiR-155	-14.10	7.423	0.145	Down regulation
MiR-202	-20.14	15.02	0.464	Down regulation
MiR-409-3P	-18.17	9.409	0.4299	Down regulation
MiR-127-3P	6.793	5.966	0.4649	Up regulation
MiR-652	-3.823	7.072	0.6915	Down regulation

*significance (p-value≤0.05) (-) indicates down regulation.
The fold regulation for control value = zero.

MiR-21 is significantly up regulated by 30 fold than control group with p-value = 0.0001 while miR-let7 is significantly down regulated by 35 fold than control with p-value 0.0160

ROC curve analyses were performed to evaluate the diagnostic power of the 10 studied miRNAs for detection of early stages breast cancer .The present results showed that among the ten miRNAs analyzed miR-21 and miR-let7c were significant in early stages breast cancer (fig. 1).

MiR-21 revealed sensitivity of 60% with AUC of 0.5667 (95%- CI [0.4059-0.7274]) with specificity 56.4% (figure A) .MiR-let 7c revealed sensitivity of 55% with AUC of 0.5119 (95%-CI [0.3586 - 0.6651]) with specificity 40% (figure D).



Figure(1):ROC curve of miR-21(A),miR-148B(B),miR-652(C) and miR-let 7c (D)

Table(2): The Expression level of miRNAs in familial high risk group:

MiRNAs	Mean fold regulation(FHR)	P-value	Changing in expression
MIR-21	-11.26	0.7645	Down regulated
MIR-LET 7C	-13.30	**0.0014*** **	Down regulated
MIR-145B	-5.712	**0.0478***	Down regulated
MIR-148B	-64.44	0.1619	Down regulated
MIR-10B	-72.05	0.0849	Down regulated
MIR-155	-3.885	**0.0014*** **	Down regulated
MIR-202	-9.932	**0.0101***	Down regulated
MIR-409-3P	-21.54	0.1616	Down regulated
MIR-127-3P	-5.477	0.0838	Down regulated
MIR-652	-41.86	**0.0478***	Down regulated

(FHR) familial high risk. *significance (p-value≤0.05)

There are significant change in the expression of miRNAs (let 7c, 145b, 155, 202 and 652) with p-value 0.0014, 0.0478, 0.0014, 0.0101 and 0.0478 respectively.

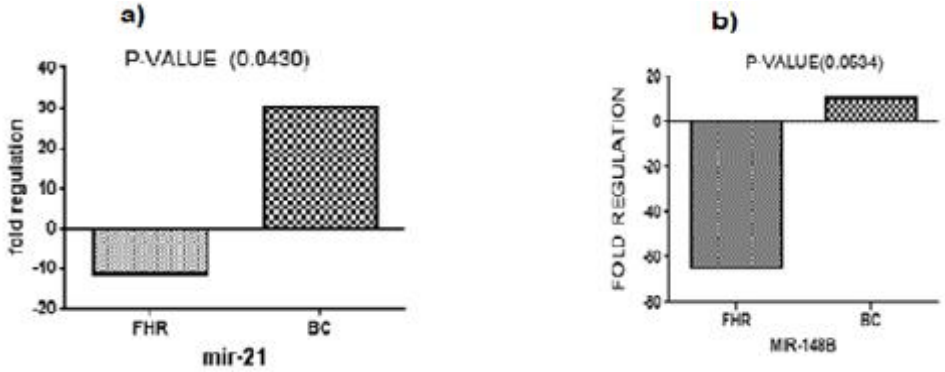
2- Analysis of gene expressions of miRNAs in plasma of BC and plasma of FHR.

In this study we analyzed expression level of different miRNAs isolated from early stages BC (n=20) compared with plasma miRNAs of FHR (n=23). Our data recorded significant differences for both miR-21 and miR-148B between early stages breast cancer and familial high risk breast cancer with p-value **0.0430 and 0.0534** respectively as shown in table (3)

Table(4): expression of studied miRNAs in plasma of BC plasma and FHR :

MiRNA	mean of fold regulation (FHR)	mean of fold regulation (BC)	p-value
MiR-21	-11.26	30.53	**0.0430***
MiR-LET 7C	-13.30	-35.63	0.9751
MiR-145B	-5.712	-1.620	0.1071
MiR-148B	-64.44	11.21	**0.0534***
MiR-10B	-72.05	24.81	0.2418
MiR-155	-3.885	-14.10	0.7041
MiR-202	-9.932	-20.14	0.5585
MiR-409-3P	-21.54	-18.17	0.7899
MiR-127-3P	-5.477	6.793	0.33
MiR-652	-41.86	-3.823	**0.0845**

*significance (p-value≤0.05)



figure(2): Represent differences in fold regulation between early stages breast cancer patients and familial high risk individuals (a): miR-21 and (b): miR-148b

Table(4): -The correlation between miRNAs (miR-21) fold regulation and different clinico-pathological factors in early stages breast cancer:

parameters	MEAN fold regulation for miR-21	P-value
Tumor size 2 (N=16, 80%)	33.46	0.0160*
Stage2 (N=17 , 85%)	30.53	0.0001***
Lymph nodes (+ve) N=12 , 60%)(	42.25	0.0130**
Positive family history (N=15, 75%)	39.71	0.0001***
HER-2 NEU (-VE) (N=18 , 85%)	30.36	0.0019**
Estrogen receptor(ER) (+VE) Bc patients N=17 , 85%)(	22.19	0.0019**
Progesterone receptor (+VE) Bc patients (N=18 , 90%)	21.15	0.0008***
Invasive ductal carcinoma Bc patients (N=17 , 85%)	34.86	0.0001***

N=numbers of patients *significance (p-value≤0.05)

Table(5): -The correlation between miRNAs (miR-let 7c) fold regulation and different clinico-pathological factors in early stages breast cancer:

Parameters	Mean fold regulation for miR-let 7c	P-value
Tumor size 2 (N=16, 80%)	-46.06	0.0013**
Stage2 (N=17 , 85%)	-40.28	0.0053**
Lymph nodes (+ve) N=12 , 60%)	37.9-	**0.3516**
Positive family history (N=15, 75%)	-30.19	0.0001***
HER-2 neu (-ve) (N=18 , 85%)	-41.42	0.0001***
Estrogen receptor(ER) (+ve) Bc patients (N=17 , 85%)	-42.45	0.0001***
Progesterone receptor (+ve) Bc patients (N=18 , 90%)	-40.02	0.0001***
Invasive ductal carcinoma Bc patients (N=17 , 85%)	-34.50	0.0053**

N=numbers of patients *significance (p-value≤0.05)

Table(6): Correlation between miR-21and breast cancer subtypes

Subtype	MEAN fold regulation for miR-21	P-VALUE
Luminal A(N=13)	24.31	0.0037*
Luminal B(N=3)	31.51	0.0001***
Triple negative(N=2)	115.0	0.0012*

N=numbers of patients *significance (p-value≤0.05)

Table(7): Correlation between miRNA let7c and breast cancer subtypes

subtype	Mean fold regulation for miR-let7c	P-value
Luminal A(N=13)	-38.74	0.0001***
Luminal B(N=3)	-4.775	0.0001***
Triple negative(N=2)	1.648	0.0012**

N=numbers of patients *significance (p-value≤0.05)

Table(8): Correlation between miR-155 and breast cancer subtypes

Subtype	Mean fold regulation for miR-155	P-VALUE
Luminal A(N=13)	-11.91	0.0001***
Luminal B(N=3)	-31.18	0.1297
Triple negative(N=2)	7.264	0.00120**

N=numbers of patients *significance (p-value≤0.05)

Luminal A: +ve ER ,+ve PR and -Ve HER-2 neu

Our results showed that miR-10b had significant up regulation while miR-155 showed significant down regulation with p-value=**0.0132** and 0.0002 respectively when correlated with positive lymph nodes.

MiR-145b and miR-148b didn't show any significant correlation with clinical pathological data except positive family history for breast cancer patients both showed up regulation with p-value **0.0168 and 0.0168** respectively.

The correlation between patients with positive family history with non-cancerous familial high risk individuals were significantly changed in miR-21 and miR-145b with p-value0.0254 and 0.0542 respectively.

MiR-155,miR-202 and miR-409-3P all had significant down regulation in patients with ER+ve, PR+ve and in luminal A subtype.

Discussion:-

The breast cancer is the most frequent cancer among women and the second leading cause of cancer death in women in Europe and North America and its incidence still increasing. Currently, mammography is the standard screening tool worldwide, but this technique is not without limitations and is associated with substantial over diagnosis as well as a high false-positive rate (Taplin et al. 2008). About 3% of the population screened annually with mammography will show abnormalities, but about 65% of these will be false positives (Langagergaard et al. 2013). In addition, small cancers are easily missed, especially in younger women due to denser breast tissue (Carney et al. 2003). Therefore, there is a need for highly sensitive and specific minimally invasive biomarker-based assays for early detection of breast cancer, alone or in combination with mammography.

This study indicates that miRNA-analyses have diagnostic and prognostic potential and could improve early stage BC detection in the future. There are several possible applications of miRNA profiling conceivable in the future. Firstly, miRNA profiles could help to reduce unnecessary breast biopsies if miRNA sets could be identified which reliably identify BC free individuals. Secondly, miRNA profiling could be used as a pre-screening method for example by general practitioners to identify women with an urgent need for breast diagnostics. Thirdly, in younger patients with dense breast tissue a future miRNA-based BC screening could possibly provide better sensitivity and specificity than the mammography even without radiation exposure.

In The current study, we profiled 10 miRNAs and use miR-16 as internal control. 4 out of 10 miRNAs were up regulated while 5/10 miRNAs were down regulated. The present study showed highly significant up regulated in miR-21 in early stages breast cancer with p-value 0.0001.

MiR-21 is one of the oncoMiRs with the highest oncogenic potential (Pan et al., 2014).It has been reported that miR-21 has been implicated in initiation, progression, and metastasis of breast carcinomas as well as a variety of other cancers by targeting various important genes (Huang et al., 2009: Song et al., 2010)In breast cancer, it has been demonstrated that miR-21 functions as an oncogene by targeting tumor suppressors genes such as phosphatase and tensin homolog (PTEN) (Huang et al., 2009), programmed cell death 4 (PDCD4) (Yang et al., 2009), tissue inhibitor of metalloproteinase 3 (TIMP3) and other tumor—related genes, and tropomyosin 1(TPM1).MiR-21 regulates its target genes in a sequence-specific base paring on their 3'-untranslated regions (3'UT R), but in an incompletely complementary way, unlike small interfering RNAs. This leads to degradation of transcripts and translation inhibition, which blocks tumor suppressor mRNAs on their way to prevent cancerogenesis. The next step is oncogenesis, and miR-21 gets its functional prefix-onco (oncomiR-21) (Rask et al., 2011).

We found significant up regulation for miR-21 in patients with ER (+ve) also for patients with PR (+ve) with p-value 0.0019 and 0.0008 respectively.

This results showed that miR-21 correlates positively with ER and PR status and might be used as a potential biomarker independent of pathohistological factors such as Ki-67 or Her-2, as well as for novel clinical stratification of patients with different ER and PR receptor status in order to steer these patients for new kinds of potential therapies such as anti-miR therapy. MiR-21 correlates negatively with the age of patients, and our results also confirmed that miR-21 reaches higher levels in younger patients (pre-menopausal) than in older patients (post-menopausal). (Qian et al., 2009) also showed that miR-21 expression negatively correlated with the age of patients (Qian et al. ,2009).

Our findings have also shown that size of the tumor has not correlated with miR-21 over expression. But, tumors with maximal diameter over 2 cm differed from tumors with maximal diameter 2 cm or less, this also according to. Iorio (Iorio et al. 2005) While Li et al showed that bigger tumors had lower miR-21 expression levels than smaller. (Li et al., 2013) which means that more detailed analysis are required.

The present results are in agreement with Huang et al (Huang et al., 2009) who showed that miR-21 over expression was related to positive lymph-node which means that expression of miR-21 increase by increase invasion and aggressiveness of the tumor.

The results showed significant correlation between expression of miR-21 and patients with (invasive ductal carcinoma, tumor size 2, stage 2 and free surgical or safety margin) Furthermore, it highly correlated with luminal A subtype (positive ER, positive PR and negative HER-2neu).

The present study showed significant differences in expression of miR-21 in breast cancer patients with positive family history and individuals with high risk which appear the importance of using miR-21 in individuals of strong family history to predict which individuals require extreme follow up than others.

According to those findings and findings from other previous study , (Petrović et al. ,2014) miR-21 is a potential biomarker for breast cancer invasion, rather than carcinogenesis and that it might be great biological marker for regrouping the patients with different receptor status.
Another examined miRNA is miR-let7c which was highly significant down regulated in early stages breast cancer with p-value 0.0160. Let-7c was not only likely to be down-regulated in breast cancer (Sempere et al. 2007), but was also found to be a tumor suppressor in prostate cancer .(Nadiminty et al., 2012).

The let-7 family members function as tumor suppressors and have been associated with various target genes, including Ras (Johnson et al., 2005), high mobility group AT-hook 2 (Sakurai et al., 2012: Zhu et al., 2009) and B-cell lymphoma extra large Bcl-xL (Shimizu et al., 2010) . Let-7 expression is down regulated in a number of malignancies. Similarly, the present study identified that let-7c expression was down regulated in BC compared with healthy controls. Thus, the present and the previous studies indicated the suppressive role of let-7 miRNAs in tumorigenesis. It is now widely accepted that let-7 levels are high in normal cells and reduced in invasive cancer cell lines. However, the mechanism of let-7 deregulation and its role in tumorigenesis is currently not fully understood.

A greater number of studies focusing on the function of miRNAs have been established, particularly those investigating the roles of circulating miRNAs in disease diagnosis (Johnson et al., 2005) To further investigate the role of let-7c expression levels in early detection of BC, the circulating let-7c levels were compared between BC patients and healthy familial high risk. It was identified that let-7c expression was lower in the plasma of BC patients compared with the healthy familial high risk.

The important role of pathological analysis in the diagnosis of BC is biomarker testing, specifically the accurate assessment of the ER and PR status of BC tissues (Sofi et al., 2012). Previous studies have revealed that let-7 is a negative regulator of ERα signaling (Sun et al., 2013), indicating that plasma let-7 levels may serve as a marker for the ER status. Previously, significant associations have been reported between tumor size in BC and ER/PR- positive rate.(Faheem et al., 2012).

In the present study there are significant correlations between the expression level of let-7c and the ER/PR- positive rate, HER-2 new negative, increasing tumor size, free safety margin, positive family history of the patients, as well as increasing stage of tumor. Subsequent investigation into the association of let-7c expression in the premenopausal patients was lower compared with the postmenopausal patients, indicating that the premenopausal status may affect let-7c expression levels.

The results agree with previous study that found let-7 levels were found to be lower in women with breast cancer that had node positive compared to node negative disease (Heneghan et al. ,2010)**.**

The current study showed significant association between expression of miR-10b and positive lymph nodes which confirm that this microRNA increases in metastatic cancer than non metastatic cancer while there are no correlations between expression of miR-10B and other parameters such as tumor size,(ER,PR and HER-2 neu) receptor or menopausal states.

Functional studies have demonstrated that miR-10b over expression promotes cell migration and invasion *in vitro*, and initiates tumor invasion and metastasis *in vivo* (Ma et al., 2007).

It was shown by our result that miR-155was significantly correlation with positive lymph nodes, patients with positive ER , patients with positive PR and patients with negative HER-2 neu . It is also significant correlate with luminal A subtype. Therefore, the miR-155 appears to play an essential role in breast cancer metastasis.

The strengths of our study are (i) the standardized processing of blood samples to generate plasma within 2 hrs of collection with a two-step centrifugation protocol, To minimize (pre)analytical variability, which could lead to bias in miRNA quantification, we standardized our blood handling, i.e., we processed all blood within 2 hrs. of collection and used a two-step centrifugation protocol. In this protocol the second step is an additional high-speed plasma centrifugation step prior to freezing, which allows the preparation of cell-free plasma devoid of cellular components and debris. Additionally, we matched our healthy controls to the investigated patients by gender and ethnicity. (ii) carrying out validation studies in a blinded manner, (iii) Investigating plasma samples which have been taken at the time-point of breast cancer diagnosis before the patients underwent any therapeutic procedures (exclusion of possible effects of therapeutic treatments on miRNA expression levels).

Conclusion:-

In conclusion, the current study showed that among the ten analyzed miRNAs, miR-21 and miR-let 7c may be potentially used as diagnostic biomarkers for early stages breast cancer. Also, both miR-21 and miR-148B may be used in the follow up of the individuals of family history as potentially early detection biomarkers. Such multimarkers blood test might provide a prescreening tool, especially for younger women to facilitate decisions about which individuals to recommend for further diagnostic tests**.**

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