E-ISSN:2349-4166
P-ISSN:2349-4158
Review Article
Cancer

Public Health Review - International Journal of Public Health Research

2018 Volume 5 Number 1 January-March

Publisher
MEDRESEARCH
www.medresearch.in

Micro RNA based cancer screening by blood test: a review

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DOI: https://doi.org/10.17511/ijphr.2018.i1.03

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Context: Circulating microRNA (miRNA) is of great importance to thebiomarker basednon-invasive cancer diagnosis. **Objective:** To identify circulating biomarkers for cancer screening and its usability with a micro fluidic device for developing an easy to use, cost effective screening tool by way of a blood test. **Method:** Reviewed published literature for expression of onco-miRNAs and miR-21(microRNA-21) in cancer patients' serum and their detection methods. **Results:** miR-21 was chosen as circulating biomarker for breast cancer screening andtesteda microfluidic device for breast cancer with 21 breast cancer and 30 healthy volunteers' blood samples, results concurred with qPCR, the gold standard for quantification. **Conclusion:** In comparison to mammogram, which is the gold standard for breast cancer screening, microfluidic platform-based blood test for screening can reach a large population at affordable cost.

Keywords: Cancer, Biomarkers, miRNA, Screening

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How to Cite this Article

Salim B, Kandaswamy A, Athira MV, Kumar MV, Adityan R Micro RNA based cancer screening by blood test: a review. Public Health Rev Int J Public Health Res. 2018;5(1):16-25.
Available From
https://publichealth.medresearch.in/index.php/ijphr/article/view/79

To Browse

Manuscript Received	Review Round 1	Review Round 2	Review Round 3	Accepted
2018-02-10	2018-02-20	2018-02-27		2020-03-03

Conflict of Interest	Funding	Ethical Approval	Plagiarism X-checker	Note
No	Nil	Yes	6%	

Introduction

Cancer accounts for the largest mortality in the developed countries, and the second largest in the developing countries, making it a global health problem [1]. In many of the cancer cases, the reason for the poor survival rate isdue to diagnosisat anadvanced stage.

Breast cancer, if diagnosed early has a greater hope of survival, still breast cancer remains the highest cause of women's mortality. Screening techniques for breast cancer recommended by WHO is mammography and clinical examination, but they are hospital centric where clinical examination or imaging technique is used [2].

The imaging technique such as mammogram pose problems when high-risk population requires to be screened every year or more often as suggested. The mammogram is not a highly sensitive technique for young women having dense breast [3]. Molecular methods are more accurate and prognostic, but affordability for most of the population remains a block.

Molecular diagnosis is quite advanced and lots of research outcomes are immature state where the miRNA is used as a biomarker. Cancer-related miRNA research has reported many miRN As being dysregulated for various types of cancer. Real time PCR is one of the methods used to quantify the expression of miRNA using molecular beacon probes.

Fluore-scence based biomarker detection using molecular beacon probes is certainly popular with biomedical applications. This review article is designed for developing a screening device for breast cancer where early detection can save a life.

Objective

MicroRNA has been in research focus for almost two decades leading to cancer diagnosis, prognosis, and therapeutics. The miRNA signature in circulation is set to revolutionize the diagnostics and prognostics. In this review article, we are exploring the possibilities of screening for cancer by blood test using miRNA as a biomarker.

Study on miRNA-18a which is a biomarker for retinoblastoma resulted in engineering a platform for screening retinoblastoma measuring over expression of miR-18a in patient's serum [4].

The work on retino-blastoma is the motivation for reviewing miRNA as a potential biomarker for screening and prognosis of other prevalent cancers. Circulating miRNAs are of great importance for non-invasive diagnosis. In this review, the authors have considered the onco-miRNA in general and miR-21 in particular, which are over expressed in breast cancer and cervical cancer.

Custom designed molecular beacon probes make it possible to screen cancer by blood test using a microfluidic device attached with a fluorescence reader. In comparison to the prevailing scheme of screening for breast cancer, which is the mammogram, microfluidic platforms-based blood test for screening can reach a larger population at an affordable cost. Circulating microRNAs are studied here to realize screening technique for cancer by a blood test.

Methods

Data Sources: Google scholar was used for searching the published research papers and review papers related to biomarkers, diagnostics, miRNA. Mendeley software was also used for searching related articles and consolidate the required articles well sorted based on content.

Study Selection: Key words used for the search were miRNA and Cancer, Role of miR-21 in cancer, Significance of biomarkers in cancer, molecular diagnostics, and onco-miRNA. Ample literature was found and those articles in English specifically related to miRNA applied as biomarker were included in the study.

However, some research papers were rejected based on the exclusion criteria such as language other than English, biomarker from Tissue and urine, and research papers published before 1990.53 research papers were shortlisted for detailed review.

Sample Collection: Institutional ethical clearance (IHEC No: 16/180 dated 07-09-2016) was obtained to collect blood samples from confirmed breast cancer patients and healthy voluntary individuals, with their consent who were in the most vulnerable age group of 25-60 for breast cancer. 5ml of blood samples were collected from twenty-one BC patients and thirty healthy volunteers as a control. Control blood samples were collected from individuals who underwent master health checkup program and were diagnosed not having breast cancer.

Data extraction: 53 research papers were examined, and the data extracted from the literature were the ones specifying the clinical significance of miRNA expression. Inclusion criteria for our study were based on identifying molecular Diagnostic methods of cancer, circulating biomarkers and Biomarkers involved in Breast cancer in particular and miRNAs in common to other cancers like Pancreatic cancer, Cervical cancer, Stomach cancer and Lung cancer.

Results

The study was completed by identifying a suitable circulating biomarker for screeningbreast cancer by a blood test using the micro fluidic platform. A matching molecular beacon probe was chosen for measuring the expression of target miRNA. A microfluidic mixer was designed and fabricated which acts as a hybridization platform for serum miRNA and the molecular beacon probe. The mixer is designed with high mixing efficiency to enhance hybridization at low reagent volume. It was proved that the simple method of reading expression level of miRNA 21 in a micro fluidic platform is sufficient for screening breast cancer and the method is minimally invasive, bias free and cost effective. Various biomarkers and methods used for detecting their expression levels are detailed below. Based on this study a novel method is evolved to screen breast cancer in a cost-effective way to address screening of mass population.

Biomarkers: A biomarker is an indicator of biological state identified with blood or other body fluids or tissues. Biomarkers are researched very much towards diagnostic applications. Any abnormal state of the biological system is found to be represented by variations in proteins, nucleic acid, antibodies etc. These variations could be referred to as biomarkers for a condition. The alterations can be due to several factors such as mutations in germ cell or somatic cell, transcriptional changes, and post translational modifications.

There are different types of biomarkers, such as proteins (e.g., a catalyst or a receptor), nucleic acids (e.g., a microRNA or another non-coding RNA), antibodies, and peptides, among different classes. miRNA has a major role in cell capacities, playing an important role in gene regulation. Currently, multiple miRNAs have been found abnormally expressed in tumor cells and closely associated with harmful cancer phenotypes.

A biomarker can likewise be a collection of alterations, such as expression of genes, proteomics, and metabolomics signatures. Biomarkers can be detected in circulation or excretions or secretions, and thus easily tested non-invasively and serially, or can be tissue-derived, and require either a sample of living tissue for analysis or special imaging for evaluation.

MiRNAs as biomarker for cancer diagnosis: miRNA sare small noncoding sequences that are unique molecules cleaved from pre-miRNA precursors. miRNAs are about 21-25 nucleotides in length, and they are partially complementary to one or more messenger RNA molecules [5]. miRNAs can use two distinct mechanisms to control protein-coding genes: "turning-off" the whole function of the target gene, and "turning-on" the expression level of multiple target genes within suitable ranges. In the earlier case, a miRNA decreases the expression level of the target mRNA at which the gene can no longer function, probably leading to observable phenotypic changes including cell death or abnormal cell phenotypes [6], [7]. In the latter case, miRNA alters the expression level of hundreds of genes to various degrees, maintaining the cellular functionality. miRNA was first discovered in Caenorhabditis elegans by a group of scientists lead by Victor R. Ambros in the year 1993. miRNA plays an important role in regulating various genes post-transcriptionally by controlling gene expression levels [8]. miRNAs are found to regulate stem cell differentiation which is important to various organ development [9]. Some miRNAs from miR-290 clusters are found to regulate embryonic stem cell cycle by targeting the cell-cycle inhibitors p21 and LATS2 thereby regulating G1-S phase transition [10]. miR-125 bplays a role in proliferation and differentiation of hematopoietic stem cells. It also regulates and activates immunity-related cells like macrophages which are mainly responsible for inflammation process and innate immunity [11]. miR-15 and miR-16 induce apoptosis in BCL2 gene (B-Cell Lymphoma) which is an apoptotic gene responsible for many types of cancers including leukemia, lymphoma [12]. miRNA, let-7 found to have a role in pathogenesis of lung cancer. Low-level of expression of let-7 was found in lung cancer cells. The increase in the expression of let-7 significantly reduced the cancer cell growth inferring that let-7 gene regulates lung cancer development [13]. Reported experimental studies showed that miR-1 and miR-13 proliferate and differentiate skeletal muscles [14].

From the overall study, it is evident that miRNAs play a crucial role in regulating important genes that are necessary for human life. At the same time miRNAs closely correlate with pathogenesis of diseases like cancer and cardio related diseases [15] that has initiated the design of diagnostics and therapeutics tools.

Clinical trials have been evaluating the relationship between miRNA expression and cancer diagnosis and prognosis. Expression profiles of many miRNAs derived from tumor tissues are useful in diagnosis and prognosis of the patients. Lu et al. demonstrated that miRNA expression profiles can be used to classify different variants of cancers, and are expressed in high amount when compared to mRNA expression profiles [16]. miR-181 family has shown an independent prognostic assessment in both cytogenetically normal and abnormal acute myeloid leukemia patients [17],,[18],,[19],,[20].

Further more, several studies have identified stable miRNAs in human serum or plasma with the distinctive expression pattern of miRNAs. Circulating miRNAs have also been shown to be able to serve as diagnostic/prognostic indicators. Differentially expressed circulating miRNAs are reported on patients of varying types of cancers including multiple myeloma, nasopharyngeal carcinoma, colon cancer, pancreatic cancer, diffuse large B-cell lymphoma, squamous cell carcinoma, lung cancer, ovarian cancer, gastric cancer, prostate cancer, breast cancer and several others [21],,[22].

Cancer causing miRNA: Onco-miRNAs are cancer-causing miRNA that are either up-regulated or down-regulated in cancerous cells. First observed cancer-causing miRNAs were miR-15 and miR-16 in B-Chronic Lymphocytic Leukemia (B-CLL) cells [23]. Frequent deletions and down-regulations of these two miRNAs were observed. Experiments revealed that there should be a constant expression of certain miRNA for a tumor progression. This was proved by experimenting with knocking of miR-21 in mice models. Activation of miR-21 increased tumor cell growth and inactivating miR-21 decreased tumor cell progression. Such a phenomenon in which tumors seemed addicted to certain miRNAs was named as Oncogene addiction [24].

Regions of miR17-92 clusters were found to be highly expressed in follicular lymphoma and B cell lymphoma and it is also found intumors including prostate, pancreas, breast and stomach cancer [25], [26].

MiR-155 contributes to the pathogenesis of cancer development which was proved using transgenic mice as a model. Mice carrying miR-155 transgene under control of Immunoglobulin E (IgE) expressed high levels of miR-155 in B cells which further caused leukemia or other blood-related cancer [27]. miR-21 targets important tumor suppressors like PTEN (Phosphatase and Tensin Homolog), PDCD4 (programmed cell death gene 4), Tissue Inhibitors of Metalloproteinases-1 (TIMP1) and TIMP3 [28]. Oncogenic role of miRNAs has been well reviewed by researchers all over the world [29].

MiR-21 is one of the frequently detected miRNAs during the profiling of miRNAs present in many types of cancer samples. In most of the miRNA studies, miR-21 is highly dysregulated[30]. It is a specific miRNA that has been upregulated in most of the epithelial cell derived cancers like breast, lung, prostate, colon cancers and hematological derived cancers like leukemia, lymphoma [23], [31], [32]. Experiments with knockout mouse models revealed that miR-21posses oncogenic function through inhibiting the cellular apoptosis[24]. Thus, a review of miR-21, its impact on cancer growth and its detection methods keeping breast cancer in focus was taken up.

MiR-21 in Breast cancer: Over expression of aparticular miRNA is not unique to one cancer. Multiple miRNA sarereported to be over expressed in a cancer and a single miRNA is found to be over expressed in many types of cancers also. Over expression of few miRNAs in some common cancers is shown in Table 1. miR-21 is highly expressed in breast cancer and it was proved experimentally [33]. miR-21 directly alters the expression pattern of PTEN genes which are tumor suppressor genes in breast cancer (BC)samples.

Table-1: List of miRNAs dysregulated in some common cancers.

Breast cancer	Pancreatic cancer	Lung cancer	Stomach Cancer
miR-21	miR-221	miR-155	miR-21
miR-155	miR-222	miR-21	miR-125b1
miR-146	miR-125b1	miR-17-5p	miR-92-2
miR-34a	miR-103-2	miR-16-2	miR-221
miR-29c	miR-100	miR-199a-2	miR-100
miR-221	miR-21	miR-215	miR-103-2
miR-181		miR-205	miR-125b1
miR-365			miR-215
miR-29b			

It was found that higher expression of miR-21 reduces the expression of PTEN [34].

This shows that miR-21 regulates the PTEN gene which causes cancer. This up-regulation in breast cancer was proved by other methods like Northern blotting, Microarray technique and in situ hybridization[35]. miR-21 expression was studied experimentally to check its expression levels in breast cancer samples. Real-time PCR studies showed that miR-21 is highly expressed in Breast cancer tissues when compared with normal tissue samples. Expression was significantly higher ($p < 0.05$) when compared with control samples. The role of miR-21 in cellular invasion was also studied with BC cell lines which reported 32% increase in the Cellular invasion in BC samples. Silencing of miR-21 showed 62% decrease in invasion activity. These results support the role of miR-21 in tumor invasion [36]. TIMP-3 is a protein that inhibits metalloproteinases which are responsible for degradation of extracellular matrix. The relation between miR-21 and TIMP3 expression was studied using Western blotting methods. BC cells with high miR-21 expression (MDA-MB-231, MDA-MB-435) and cells with less expression (BCAP-37, MCF-7) were used in western blotting analysis. Results showed that TIMP3 protein levels were reduced in BC cells with higher miR-21 expression. And it was higher with BC cells having a low level of miR-21 expression. The negative relation was seen between TIMP3 and miR-21 in this study. Serum samples of BC patients were analyzed for miR-21 expression using the RT-PCR method. Expression of miR-21 was found higher (30.82) when compared with healthy controls (9.1). Calculated area under ROC curve for miR-21 expression was 92.9% [37]. Thus, miR-21 which was expressed highly in BC tissue and blood samples is a suitable biomarker for detecting breast cancer.

MiR-21 in Cervical cancer: Cervical cancer (CC) was reported to be world's fourth leading cancer that leads to mortality in women [38]. Cervical cancer develops into malignant through the premalignant lesion. It takes ~10 years for epithelial cells to change to cervical intraepithelial neoplasia and invasive cervical cancer[39]. The report shows analysis of many cervical cancer samples using RT-qPCR studies, where miR-21, -29a, -200a, -25, -486-5p were found to be highly expressed when compared to control samples. Expression levels of miR-21 at different stages of cervical cancer also varied. It was progressively higher from the initial stage to final stage of CC. Fold change of miR-21 was found to be 2.02 [40].

Luciferase reporter assay was performed with CC sample transfected with anti-miR-21 and it was observed that PDCD4 gene was up-regulated. Similarly, RASA-1 (RAS p21 protein activator-1) was also found to be up-regulated. These results showed that miR-21 alters the expression pattern of both PDCD4 and RASA-1[41]. RASA-1 gene directs in the formation of protein RasGAP which regulates RAS/MAPK pathway. This pathway is responsible for Normal cell functions. miR-21 reduces the expression levels of RASA-1 thereby deactivating the important cellular function. This enables the invasion of cancer cells to other organs[42]. MTT assay demonstrated that inhibition of miR-21 reduces tumor cell proliferation and Annexin V staining demonstrates inhibition of miR-21, increased apoptosis of tumor cells. From the above studies, it is learned that miR-21 plays an important role in the pathogenesis of CC. As it is highly expressed in cervical cancer, it can be a used as a biomarker for detecting and diagnosing cervical cancer.

MiR-21 in Pancreaticcancer: Pancreatic cancer is a lethal malignancy with increasing incidence and a very high mortality rate [43]. The miRNA expression pattern shows a noticeable difference in normal pancreas and chronic pancreatitis 44. The cell cycle checkpoints and kinetics have a major role in regulating cell proliferation. Various studies in pancreatic cancer have shown several oncogenic miRNAs is negatively affecting tumor-suppressor genes that act as regulators of the cell-cycle progression. One of the most often studied miRNAs, the miR-21 affects a tumor-suppressor PTEN whose protein product prevents the spread of tumor cells and controls the frequency of cell division. The over expressed miR-21 attaches to the mRNA of PTEN, thereby it reduces tumor-suppressive function [44]. Many of the miRNA expression level that is elevated in endocrine pancreas cancer and pancreas adenocarcinoma and is similar, including miR-221, -100, -125b and -21 [45]. Some miRNAs play a role in the efficacy of anticancer therapy and thus present themselves with new therapeutic possibilities. The Nano-molar concentrations of antisense miR-21 oligonucleo-tides effectively inhibit their target oncogenic miR-21 and it can reduce proliferation of pancreatic cancer cell lines and, along with gemcitabine, prevent their growth.

MiRNA Detection methods: The data presented above indicated that miRNA expression level is a good indicator to identify the presence of cancer.

The scientific methods which are popular and well in use are briefly explained.

Northern Blotting: Northern blotting method is one of the common methods for studying the gene expression by detecting DNA or RNA in each sample. It is a widely used method that does not require any complex equipment and high knowledge, and also the facility is commonly available in most of the research laboratories [46]. Agarose gel containing fractionated DNA or RNA is transferred into membrane support. Blots can be found in the membrane indicating that RNA has been transferred to the membrane. Specific probes are labelled with membrane and the hybridized probes will emit chemiluminescence which can be detected using a detector[47]. A number of miRNAs were detected and characterized using this method. miRNA biogenesis was well studied by the length of miRNAs detected in the membrane using mouse model[48]. Disadvantage of this technique is that probe specific DNA or RNA are poor in sensitivity and also time consuming. However, researchers have found out a new protocol for precise detection with high sensitivity which includes extraction of RNA, separation by gel electrophoresis and hybridization and detection of locked nucleic acid (LNA) modified oligonucleotide probes. The use of LNA allows high sensitivity and specificity for detection of specific miRNAs.

Real-Time PCR (q-PCR): Real-Time PCR method is based on the detection of fluorescence produced during the amplification of DNA or RNA target. This method is used for qualitative assays. miRNAs can be quantified and validated using this method. miRNAs and their precursor miRNAs were detected in the experimental studies. Commercial miRNA Assay kits are available in the market (Exiqon, Qiagen) etc. The copy number of the given DNA or RNA can be quantified based on the expression level [33]. Forward and reverse primers will be used to amplify the target DNA. In the case of micro RNAs, its sequence can be retrieved from miRNA registry[49]. Profiling of miRNAs that are associated with diseases and cellular functions can be done using this method [50], [51].

Microarray Technique: Micro RNAs are detected based on its expression in a cell or sample. Microarray technique is one of the methods that precisely measures the miRNA expression levels in a sample. miRNA microarray contains a large number of miRNA probes printed on it.

It can simultaneously detect the expression of various miRNAs in a single sample. Application of microarray technology in detecting miRNA expression gives accurate results when compared to other methods like northern blot, cloning, membrane arrays using radioactive detection[51]. Acute Myeloid Leukemia (AML) was studied using microRNA microarray chips (Affymetrix) and determined the gene expression level of miR-18a. This miRNA was highly expressed and due to its expression, genes like transcription co-regulator (ID1) which prevent hematopoietic differentiation, Erythroid differentiation suppressor gene (FLI4), and Transcription factor (TCF4) were dysregulated. The positive relation between miR-18a expression and TCF3 which regulates homeostasis of hematopoietic cells was also observed [18]. Cancer related miRNAs (oncomiRs) and cardio-related miRNAs (cardiomiRs) were extensively studied and validated using microarray studies [15]. Currently, disease-related miRNAs including oncomiRNAs were studied and validated using microarray technology. Biomarkers for various types of cancer can be detected using this technology[18] and this makes it one of the therapeutic tools for diagnosing cancer and other related diseases.

Future technologies: Other than blotting methods, PCR and microarray technologies, recently researchers are developing new technologies for detecting microRNAs which are portable and time saving. Nanopore technology was developed by a group of researchers which incorporates silicon nitride membranes. Nano pores of approximately 3nm diameter were drilled and current is applied. When miRNAs with hybridized probe passes through these pores, variations occurring in current signal are detected [52]. One of the major advantages over PCR and hybridization methods is that sequence similar miRNAs (difference in one nucleotide) can be detected distinctly as a separate current signal whereas, in the other methods it is not possible.

Due to its high specificity, miRNAs having similar sequences can be discriminated and studied individually. Micro fluidics technology is also used to develop a device for miRNA detection [4]. This device consists of microfluidic mixing channel. miRNA containing samples and its specific probe could pass through the channel. Hybridization occurs due to proper mixing inside the channel and hybridized complex are detected using custom-designed fluorescence detector.

This device is portable and can be used as a diagnostic tool to detect various miRNAs with minimum quantity of sample. Biosensors are used for miRNA detection. Complementary sequence probe of target miRNA is immobilized in the sensor and sample is passed through the sensor. During base-pairing, fluorescence signal is detected and based on this detection, miRNAs can be discriminated [53].

Discussion

In the case of breast, miR-21 is highly expressed and it is proven by major experimental studies, like RT-qPCR, microarray analysis, northern blotting studies, in situ hybridization studies and in silico studies. This leads to the idea that miR-21 can be used as a biomarker for screening breast and cervical cancer. Detecting the expression level of miR-21 is not only useful for diagnosis, but also can be a guideline for therapeutic regimes. Reduction in the expression level of miR-21 on post medication may indicate a reduction in intensity of cancerous growth. A study was conducted on 21individuals in the age group of 25 to 60 who were diagnosed with breast cancer. In addition, 30 healthy individuals of the same age group who were chosen from the master health check-up program were included in a control group. Written consent was taken from all the participants. The study conducted on 21 breast cancer patients and 30 healthy volunteers, revealed that the miR-21 levels were marking difference for healthy and breast cancer samples, a difference for stages of cancer, reduced miRNA level for those who have undergone surgery as depicted in Fig.1. miR-21 level for healthy volunteers were below 70 a.u and various stages of cancer showed higher levels of miR-21.

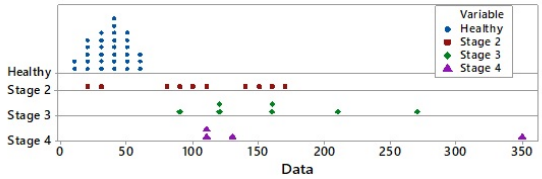

Figure-1: miRNA level as fluorescence intensity (arbitrary units) for stages of breast cancer measured on Micro fluidic platform using molecular beacon probe for miRNA 21 to target miRNA21 from serum.

Two breast cancer patients diagnosed at Stage 2 had undergone surgery while blood samples were collected, and their miR-21 levels were low as seen in the dot plot.

Conclusion

Screening for breast cancer is always challenging since the methods adopted for the same are mammogram and clinical examinations. Both are having the drawback of bias in detection based on the age of the individual. Mammography has an added drawback of radiation hazard for high risk population since the frequency of the scan is high. Thus, a minimally invasive blood test would be very much appreciated for the purpose if the same is cost effective. Microfluidic platform will help in detecting the over expression of micro RNAs with a minimum amount of sample (serum) in very less duration. Fluorescent probe (Molecular beacon probes) which is complementary to the target micro RNA is used for detection. Fluorescent probes can be designed for detecting any miRNA. This method of assessing miRNA expression will be useful even during the therapy to decide the treatment regime. Miniaturization and advancement in fabrication technology support development of a micro fluidic platform for mixing and hybridization. By modifying the micro fluidic platform to incorporate multiple probes will enable screening for multiple cancers with the same miRNA or eliminating false positive or false negative situation. Molecular beacon probes also can be designed with multiple miRN As to detect multiple disease situation, where identified miRNAs exist. Screening of high risk population of breast cancer is the need of the day and the technique should be able to reach a large number at an affordable cost.

Clinical Significance: Screening by miRNA expression using microfluidic platform remarkably makes a difference to save lives of women. The present scenario of women approaching a hospital upon a significant symptom to get an advanced breast cancer stage diagnosed is the root cause of failure in controlling cancer deaths. This method should be much useful and applicable to screen large population on point-of-care mode without any side effect. As seen from Table 1, multiple miRNAs are over expressed in a disease and a miRNA is over expressed in multiple diseases. Custom built LNA with multiple biomarkers could be used for eliminating false positive or false negative in a single blood test.

List of abbreviations

B-CLL- B-Chronic Lymphocytic Leukemia, **BCL– B-**Cell Lymphoma, **AML–** Acute Myeloid Leukemia,

MiRNA–Micro RNA, **PTEN–** Phosphatase and Tensin, **PDCD4–** Programmed Cell Death gene 4, **TIMP –**Tissue Inhibitors of Metalloproteinases, **RAS–** Rat Sarcoma, **RASA-1** –Rat Sarcoma Protein Activator-1

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