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Review Article

Identification of non-invasive biomarkers for early detection of breast cancer: A Review

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Abstract

Despite decades of research, diagnostic tests with specificity and accuracy for early breast cancer are yet unavailable. Major problems associated with poor diagnosis are either due to incompetency of reported biomarkers or small volume of patients under study. Moreover, heterogeneity of the disease further complicates the struggle of identifying effective biomarkers. Therefore, to improve the survival rate, look for new, sensitive and specific biomarkers for early breast cancer diagnosis is need of hour. In this study, we have reviewed recently reported serum biomarkers and categorized them based on their biomolecular nature such as protein, ctDNA, epigenetics regulation and miRNA. Potential role of these available biomarkers in early diagnosis of breast cancer has also been discussed. Based on the facts obtained from literature review, it is revealed that using any individual biomolecule as a biomarker is not sufficient to diagnose breast cancer at early stages rather it is suggested that a panel of proteins or miRNAs would offer better sensitivity and specificity. Whereas, unavailability of a potential ctDNA and epigenetics regulation candidate for diagnostic purpose is and suggest the use of more sophisticated techniques to unwound these regulations in serum especially at early stages of breast cancer.

Keywords: non-invasive biomarkers; breast cancer; biomolecular nature

1. Introduction

Breast cancer is the second major cause of mortality and morbidity among women across the globe. There is not a single factor which is responsible for onset, development and progression of cancer. Various genetic and environmental elements are involved in its initiation and advancement.[1] Along with various genetic determinants, non-heritable genetic factors also play an important role in the onset and progression of breast cancer. These non-heritable factors called epigenetics which may either prompt hushing of tumor silencer qualities or oncogenes activation.[2] challenges in breast cancer diagnosis and treatment are primarily attributed to its heterogeneous subtypes which results in variable phenotypic expression, progression and treatment against the malady.[3] Breast cancers are primarily carcinomas, a type of cancer in which skin cells or the cells responsible for lining of internal organs like tissue and kidney are involved. On the basis of severity of disease and symptoms, breast cancer is classified into different stages: 1)-primary tumor, very first stage of breast cancer in which a cell has undergone mutation and became a cancerous one, this stage is undetectable 2)-lymph nodes, this is the second stage in which a lump (static mass of cancerous cell) can be feel or observed in the peripheries of breast tissue, mammography or biopsies are usually performed at this stage and 3)-metastases, is the most advance stage of breast cancer progression in which tumor cells have lost their inter-cellular contact with each other and have travelled to other body parts through circulatory system, treatment at this step is highly expensive and usually in effective. Moreover survival rate of patients at this stage is extremely poor. Early detection ensures 5 years survival rate in large proportion of breast cancer patients (98%) as compared to late diagnosis in which observed survival rate (5 years) is only 23%. therefore early detection is crucial for improving the survival rate in breast cancer patients.

Till now, mammography is a gold standard for early breast cancer diagnosis. However, there are various controversies associated with this type of diagnosis. It is restricted to the location of bigger tumors, bringing about the disregard for littler tumors which limits its diagnosis ability for timely breast cancer diagnosis.[4] Moreover, at initial stages, cases of over-diagnosis of breast cancer and risks of false positive results using mammography have also been reported.[5] Furthermore, poor sensitivity for detecting breast cancer from a denser breast tissue also limits the utilization of mammography. Magnetic resonance imaging (MRI) resolves latter problem and offers better imaging of a denser breast.[6] However it is an expensive technique and there are high incidence of false positive results (37%-100%), which requires follow-up investigations and unnecessary biopsies that makes it non-specific and unreliable technique for breast cancer diagnosis.[7] So far, Screen-film mammography (SFM) is reported for optimal diagnosis of breast cancer in women older than 50 years of age with confounding false-positive and false-negative results.[8] Now scientists are using various technologies to identify biomarkers for breast cancer diagnosis at early stages. They are aiming for a specific bio-entity such as protein, circulating tumor cells (CTC), micro-RNAs, extracellular vesicles, or circulating tumor-derived cell free DNA (ctDNA). Despite of using various approaches there are several issues including tumor heterogeneity, its diversity and plasticity which hinder

the development of a sensitive and reliable biomarker. Moreover, deterioration of sample during collection and storage and unavailability of robust techniques for validation of biomarker further complicates the search and make it a challenging endeavor.

Tissue and blood are the primary sources of breast cancer biomarkers however utilization of tissues offers invasive and surgical protocols which usually compromise patient compliance. Moreover, a small piece of tissue is harvested for diagnosis which includes the operator biasness and neglects the whole bigger picture required for reliable diagnosis. Therefore, a noninvasive biomarkers with sufficient sensitivity and specificity to distinguish required treatment against heterogenous disease is of immense need.[9] Biological fluids are the potential non-invasive biomarkers for the diagnosis of breast cancer at early stages. These included urine, blood, sputum and serum etc. containing large amount of cell free biomarkers as well as greater amount of circulating genetic material to be assessed.[10] Among the biological fluids, blood is considered as a profound candidate for identification of early stage detection of breast cancer biomarker.

Blood biomarkers are superlative candidates for cancer screening as their role could be easily extended from cancer risk assessment to evaluation of applicable treatment followed by recurrence monitoring. Furthermore, it is a rich source of several cellular elements which reflects the health status of an individual and offers non-invasive cancer diagnosis. Comparing to the heterogeneous nature of breast cancer, few biomarkers have been identified for breast cancer diagnosis which either evaluates the prevalence of hormone receptors (HR) including estrogen receptor and progesterone receptor, presence of multiple copies of epidermal growth factor receptor-2 (HER-2) genes, or higher expression rate of HER-2 protein.[11] On the basis of these targets breast cancer has been classified into four different subtypes: luminal A (HR+), luminal B (HR+, HER+), HER+ and triple negative breast cancer (TN breast cancer) also known as basal-like breast cancer subtype.[12] Previously, immune-histocompatibility (IHC) tests were performed on tissue samples i.e. bone marrow and lymph nodes, to identify metastasis in breast cancer. However, the technique is expensive, time consuming and sometimes unreliable. Using blood samples for the detection of circulating tumor cells (CTCs) offers a better approach for diagnosing metastasis in breast cancer as it is quick, simple and non-invasive.[13] Quantifying mRNA levels of breast cancer specific biomarkers through RT-PCR is the most promising approach for diagnosis of metastatsis stage and a lot of improvements have been made in the technique i.e. RT-MLPA which offers effective diagnosis of multiple cancer related genes in a single cancerous cell using blood sample.[14] Although there is a lot of literature reported on various blood based biomarkers, there is not a single blood-based diagnostic test available for early cancer diagnosis and subsequent treatment against the malady. Therefore search for identification of a sensitive, specific and reliable biomarker is still in progress. Blood can be fractioned into plasma and serum, and higher sensitivity in biomarker detection with serum over plasma is already reported.[15] It might be due to higher metabolite concentration in serum. Moreover serum is pure from various proteins hence offers better resolution, sensitivity and specificity.[15] Therefore in this study, we have highlighted various types of serum biomarkers such as proteins, miRNAs, circulating DNA and epigenetic alterations for early breast cancer diagnosis. The aim of this literature review is to collect data reported on serum based biomarkers for early diagnosis of breast cancer, and to highlight their limitation and suggestions for improvements in biomarker identification.

2. Breast cancer through genes from serum

There are several circulating tumor-based cell free DNA (ctDNA) in serum which can help in diagnosis of the disease and concomitant stage at the time of discovery. There are two ways in which nucleic acid can enter in the circulation. It might be the result of cell damage followed by release of short stretches of nucleotides (i.e. < 200bp long) or nucleic acid might be directly released from tumor cells which are variable in length (upto several kilo base pairs).[16] Ratio of larger fragment over smaller fragment is termed as integrity index and value of integrity index is greater in breast cancer patients as compared to healthy controls.[17] Therefore, circulating DNA (ctDNA) could be effectively used as potential serum biomarkers. Umetani and his group revealed that serum DNA integrity is an excellent biomarker for early breast cancer diagnosis. They observed that compared to healthy females, higher DNA integrity in breast cancer patients with stage II, III and IV, respectively was reported. AUC value for discriminating healthy and cancerous patients was 0.79 and integrity of serum DNA was positively associated with invasive and metastatic stage of breast cancer.[18] To discriminate between BREAST CANCER patients with benign and malignant tumor, scientists have identified serum glyceraldehyde-3-phosphate dehydrogenase (G3PD) gene as potential breast cancer biomarker.[19] They found higher levels of G3PD in sera of breast cancer suffering from benign or malignant tumors than normal controls. Moreover, there are higher levels of circulating serum G3PD gene in patients with benign as compare to patient with malignant breast cancer. This correlation might have a diagnostic value for differentiating between benign and malignant breast cancer tumor.[19] On contrary, Roth and his group reported that ctDNA levels in serum are higher in case of breast cancer but there is not significant change in their level in case of benign or malignant tumor. Therefore, it is not helpful in predicting the breast cancer stage however nucleosome levels in serum give a useful insight for the prediction of breast cancer stage as patients positive for lymph nodes have its higher compared to node-negative patients. In accordance, their levels are even higher for metastatic breast cancer.[20]

For ctDNA biomarkers identification plasma samples are preferred over serum, because serum preparation leads lysis of several cells leading to false increase in integrity index. The false positive results are particularly seen in case of stored serum samples. However, research is still in progress to measure ctDNA from both serum and plasma through various biological elements such as ALU, LINE and SINE sequences as these are distributed in whole genome hence offer reliable diagnosis. Moreover, sophisticated techniques like PCR (beads, amplification, magnetics and emulsion), Next generation sequencing and massively parallel sequencing are being used for better identification of serum based ctDNA for early breast cancer diagnosis.

3. Breast cancer diagnosis through proteomic analysis from serum

A lot of proteome analysis has been carried out on breast cancer serum samples for the identification of potent diagnostic biomarkers. Several studies emphasized the CEA and CA15-3 as potential protein based serum biomarkers for breast cancer. These wo proteins have also been recommended by European group of Tumor Markers with low specificity and sensitivity.[21] Therefore, search of sophisticated protocols for the identification of more reliable and sensitive serum biomarkers is still ongoing. Here, we have discussed few serum proteins which are recently reported for their potential to be used as breast cancer biomarkers.

3.1 AGR3

AGR3 is a protein belonging to disulfide-isomerase family and has extensively studied as serum biomarker for cancer diagnosis .[22] Its elevated expression at mRNA and protein level is reported in breast cancer patients as compare to non-cancerous individuals; whereas expression level varies with respect to cancer subtype. [23] Surprisingly, AGR3 levels are considerably higher in luminal tuype as compared to TN type, and expression level positively correlates with G1 (low grade, tumor cells are well-differentiated) and G2 (intermediate grade, tumor cells are less differentiated) grade of breast cancer.[22] Garczyk S, and his colleagues

identified higher levels of AGR3 from sera of low grade patients compared with healthy controls through ELISA and unveil the potential of AGR3 to be used as early breast cancer diagnosis biomarker.[22] Additionally, they reported improved breast cancer diagnosis efficiency by combinatorial performance of AGR3 and AGR2 proteins.

3.2 ApoC-I

Later on, another protein reported as potential breast cancer serum biomarker was ApoC-I. It is an apo-lipoprotein, which are lipid carriers and play a vital role in lipid metabolism. ApoC-I is a small polypeptide (57 amino-acid long) and it constitutes circulating LDLP (low density lipoprotein), IDLP (intermediate density lipoprotein), HDLP (high density lipoprotein) and chylomicrons. [24] Although ApoC-I is responsible for impairing the clearance of LDLP by hindering their uptake by liver, levels of ApoC-I are decreased in breast cancer patients as compared to the healthy individuals.[25] Moreover, protective role of ApoC-I against tumor progression is reported in nude mouse models which make it a potential candidate for cancer therapeutics but exact mechanism for its down-regulation in breast cancer patients and its suppressive role in cancer progression is still not known.

3.3 Vitronectin

Vitronectin, an adhesive glycoprotein involve in cell adhesion, inflammation and blood coagulation is also reported as a promising serum biomarker for breast cancer diagnosis. Vitronectin is known to play an important role in tumor growth via angiogenesis.[26,27] In 2016, Hao and its group extensively studied the serum levels of vitronectin at various stages of breast cancer. They figured out the potential of vitronectin as an early biomarker for breast cancer diagnosis as well as its surprising role in differentiating various stages of breast cancer (0, I and II) and identifying non-cancerous, benign and precancerous lesions. Vitronectin expression and advance stages of breast cancer show a negative correlation with each other. Moreover, they reported that use of vitronectin in combination with CEA and CA15-3 can improve the sensitivity and specificity of breast cancer early diagnosis.[28] AUC value of vitronectin is 0.73 whereas, in combination with CEA and CA15-3 the value increased upto 0.83 which makes the trio an appropriate biomarker for early breast cancer detection.

3.4 Pleiotrophin

Pleiotrophin, a multifunctional growth factor, is another promising serum biomarker protein for breast cancer diagnosis. Its overexpression is reported in breast cancer cells whereas, its positive role in angiogenesis is observed in rabbit corneal assay.[29] Interestingly, a truncated version of pleiotrophin is reported which behaves antagonistically to the intact protein and inhibits tumor progression.[30] Pleiotrophin expression in sera of breast cancer patients is found to be higher than normal controls. Ma and its coworkers revealed the fact that higher pleiotrophin level in serum of is due to the release of protein from tumor cells, therefore pleiotrophin levels increases concomitantly with cancer stages.[31] Pleiotrophin expression in breast cancer stage III and IV are significantly higher than stages I and II, however difference between pleiotrophin levels in stages III and IV are not much significant. Therefore, its level could be utilized for distinguishing early and late stages of breast cancer. AUC curve for serum pleiotrophin is 0.87 which shows its specificity and sensitivity for early diagnosis.

3.5 Trefoil factors

There is another protein, trefoil factors which are small peptides secreted in GIT (gastrointestinal tract) by epithelial mucus cells. They are highly stable towards proteolytic degradation due to their unique structure and constitute three members i.e. TFF1, TFF2 and TFF3.[32] Ishibashi and his group have recently suggested role of these proteins as effective biomarkers for early diagnosis of breast cancer. Although up-regulation of TFF1 and TFF3 was observed in patients's sera, TFF2 expression level was down-regulated. AUC curve of three serum trefoil proteins was 0.96 which is highest among reported protein based biomarkers hence, makes it a powerful candidate for breast cancer screening. [33,34]

Despite of achieving higher AUC values research for identifying more powerful serum biomarkers is still in progress due to insufficient diagnosis ability of reported biomarkers. It is believed that utilization of a group of protein as breast cancer biomarker would be more effective and provide better diagnosis spectrum against the heterogeneous disease. Scientists are using MS-based protein profiling of breast cancer patients in order to identify more reliable peptides which have a significant role in breast cancer diagnosis and by the wise combination of such peptides only, a potent, reliable and sensitive biomarker could be developed.

4. Epigenetics and early diagnosis of Breast cancer

Epigenetic alterations are abnormal changes including methylation, post translational modification (PTM) to histone and nucleosome rebuilding. Among all these, methylation has profound effect on the gene regulation mechanism. DNA methylation is a process known as the addition of methyl group at CpG site within the mammalian DNA driven by proteins or enzymes.[35] DNA hypomethylation is the ubiquitous feature might prompt the regulation of oncogenes or proto-oncogenes. It is also involved chromosomal aberrations like change in recombination rate and loss or inactivation of X-chromosome. DNA hypermethylation is habitually connected with quality constraint and genomic unsteadiness (through quieting of DNA fix qualities) and can prompt the concealment of tumor-silencer qualities and compaction of chromatin.[36,37]

The various methods are used to detect the epigenetic biomarkers such as genome wide sequencing, microarray profiling. On the other hand, the specific and accurate pattern of DNA methylation is evaluated with locus specific assays like quantitative methylation of specific PCR, one advance methylation specific PCR, light-methyl assay and pyrosequencing technique. These techniques can identify the methylation at known loci with high accuracy. [38,39]

Novel analytic and prognostic biomarkers are critically expected to help in the counteractive. The discovery of abnormally expressed molecules showed during carcinogenesis can fill in as a rule for clinicians to make suitable decisions dependent on anticipated factors, for example, the probability of metastasis, tumor repeat, and life expectancy of patient.[40] So, DNA methylation based epigenetic biomarkers can be proved as promising targets for the early diagnosis of different malignancies.[9]

4.1 Non-invasive epigenetic biomarkers in serum for early breast cancer diagnosis:

Serum is the best medium to be assessed for the detection of cancer development as it is easy and simple to obtain it through non-invasive process. Serum contained large amount of cell free circulating DNA which can be analyzed to check different methylation pattern. Several researchers has been reported tumorigenesis related methylation pattern of many genes in serum with different success rate.[41]

For the early detection of breast cancer, a prospective study on biomarkers has been reported where the serum from 141 females with metastatic breast cancer showed higher methylation level in the panel of genes like HOXB4, KR1B1, RASGRF2, RASSF1, TM6SF1 and HIST1H3C.[42]

Yamamoto et al. built up an efficient method (one-advance methylation-specific polymerase chain response) for the identification of DNA methylation in serum sample of females with primary breast cancer, metastatic breast cancer and healthy control. They observed the methylation at promoter region of gene of interest (RASSF1A, GSTP1 and RARb2). Additionally they found the higher sensitivity of these biomarkers in early stage of primary breast cancer in contrast to the traditional breast cancer biomarkers like CA15-3 and CEA.[38] A panel of

genes such as hMLH1, PCDHGB7, HOXD13, P16, SFN and RASSF1 was designed by Shan et al to check the DNA methylation in serum. Methylation at promoter region in this gene panels was correlated to patients having familial cancer and inversely correlated with ki-67.[43]

4.2 Post-translational modification of histones DNA methylation and its role in breast cancer

The PTM of histones such as methylation, acetylation, phosphorylation, ubiquitination, sumoylation, and poly- ADP-ribosylation driven by histone acetyltransferase and histone deacetylase (HDAC).[3] however, recently a distinct pattern of PTM of histones have been reported in breast cancer. For example, in ER-negative breast cancer the enzymes HDAC and DNA methyltransferase are involved in inactivation of ER gene due to their presence in promoter region of the gene.[40,48]

5. Breast cancer diagnosis through micro RNA from serum

For effective management of breast cancer, quick and sensitive biomarker is required for diagnosis. There are various types of biomarkers are available microRNA found in blood, for diagnosis, stage of cancer evaluation as well as used for therapy. MicroRNA is potentially being used for diagnosis and treatment.

microRNAs are normally produced by human body but it is expressed irregularly in case of cancer but for breast cancer it is required to get a disease specific microRNA.[49] MiRNAs are single stranded short nucleotide (19-23) sequence obtained from almost 70 nucleotide, which play crucial role in post-translational modifications by regulation of gene expression in a biological system.[50-52] In human, a single miRNA can target the hundreds of mRNAs having the binding site at 3' untranslated region.[53] miRNAs not only regulate the post translational modifications but also gene expression by binding to exons including other regions of gene.[54,55]

Deregulated miRNA is prominent in case of breast cancer which acts as a signature between normal and abnormal cell proliferation especially in case of malignant breast cancer.[56] The studies suggest there are two miRNAs, miR-21 and miR-210 are up regulated in early cancer cells.[57] The study also suggested that set of nine miRNAs (miR-15a, miR-18a, miR-107, miR-133a, miR-139-5p, miR-143, miR-145, miR-365 and miR-425) can discriminate between early breast cancer and healthy one. Among them, four (miR-15a, miR-18a, miR-107 and miR-425) were up regulated while expression of remaining five miRNAs (miR-133a, miR-139-5p, miR-143, miR-145, and miR-365) were down-regulated as compared to controls. This signature was found to be 84% sensitive.[58] There is potential role of miR-10 b which is up regulated in metastasis and invasion while it is down regulated in primary breast cancer it can be called onco-miRNA.[59] In case of TN breast cancer there is signature of four miRNAs reported (miR-18b, miR-103, miR-107 and miR-652) that could predict tumor relapse and overall survival which is higher for reoccurrence of cancer and the authors suggested that any of these microRNA can be used to evaluate the TN type because of strong prognosis.[60] miR-625 is highly prognostic biomarker for stage I and II of breast cancer and correlated to TP53.[61] Another study has provided the set of five miRNA (miR-1246, miR-1307-3p, miR-4634, miR-6861-5p and miR-6875-5p) for detection of early breast cancer. This combination had sensitivity of 97.3%, specificity of 82.9% and accuracy of 89.7% for breast cancer. For early breast cancer detection the sensitivity was 98.0%.[62]

Although a number of microRNA that can be used as potential biomarkers are being reported but there is not enough abundance and there is no single method to detect such level of microRNA. To get the microRNA as biomarker there must be such technique which would be rapid, sensitive and selective for detection of microRNA in serum.

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