Review Article:

Breast cancer diagnosis – role of biomarkers

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ABSTRACT

Current routinely used serum biomarkers have limited usefulness for diagnosis and screening of breast cancer. Triple test is an accurate diagnostic test for breast cancer based on which treatment is initiated. However, the accuracy of mammography and fine-needle aspiration cytology (FNAC) when applied alone is less. Also, each of the test has its own limitations. This review attempts to highlight the current and novel markers available for diagnosis of breast cancer with special emphasis on the role of biomarkers in breast cancer diagnosis.

Key words: *Triple test, Diagnosis, Breast cancer, Biomarkers*

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INTRODUCTION

Breast cancer is still one of the leading cause of cancer related mortality in women worldwide. It represents 30% of all the cancer cases in females and accounts for 14% of all cancer deaths among females.[1] The high prevalence report is the result of better diagnostic technologies. Also, there has been a sustained decline in mortality rates over the last decade due to the increased application of effective adjuvant medical treatment. Regular screening and better awareness have resulted in a shift towards early detection of breast cancer.[2] This review attempts to highlight the current and novel methods available for diagnosis of breast with special emphasis on the role of biomarkers in breast cancer diagnosis.

Diagnosis of breast cancer

The commonest clinical presentation of breast pathology is a breast lump which may be either malignant or benign. A definite diagnosis of breast lump is important to decide on final treatment.[3] In patients with a palpable breast lump, triple test is a very useful diagnostic tool to detect malignant breast tumours. It is a combination of three tests which include clinical examination, radiological examination (mammography) and pathological examination. Clinical breast self-examination will not diagnose the breast lump accurately whether it is benign or malignant. Its accuracy is only 70% whereas accuracy of mammography and biopsy are only 82% and 78% respectively.[3] A clinical judgement of breast cancer should be supported by specialized investigations. Various screening and diagnostic modalities like clinical breast examination, mammogram and pathological examination are available. Breast self-examination forms a part of regular screening. No beneficial effects of screening by self-examination has been observed; breast self-examination had increased false positives in terms of increased numbers of benign lesions.[4] Mammographic screening is less sensitive in case of denser breast seen in younger women of age less than 50 years which reduces the ability to detect early lesions that has low

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positive predictive value, thus reducing the sensitivity and specificity of mammography in younger patients. It is not suitable for detecting small-sized tumours, as well as node-negative early-stage (T1N0) primary breast cancer (PBC) and ductal carcinoma-in-situ (DCIS) in patients with higher breast density.[5] A recent large prospective study[6] from Canada estimated that 22% of mammography screens overdiagnosed invasive breast cancers. Biopsy helps in cytological evaluation which may require local anaesthesia. It is an invasive procedure and hence is associated with morbidity.[7] Due to the limitations in each modality of the triple test, there is a need to introduce new diagnostic modalities like serum biomarkers for breast cancer diagnosis. This can potentially be improved by pairing each of the modality with minimally invasive serum circulating biomarkers.

Biomarkers in breast cancer diagnosis

A biomarker is defined as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease".[8] A biomarker should be strongly correlated with disease risk and should be used in monitoring treatment. Currently there are no markers for screening and diagnosis of breast cancer but few markers like carcino-embryonic antigen (CEA), carbohydrate antigens (CA) (CA 15-3), CA 27-29 are being used in surveillance and metastasis.[9] Other biomarkers have been identified as predictors of breast cancer risk and have shown their clinical utility in breast cancer patients. These include CEA, CA 15-3, CA-27-29, oestrogen receptor (ER) and progesterone receptor (PR), human epidermal growth factor receptor 2 (HER-2). Many other biomarkers which are associated with breast cancer including anti-inflammatory markers, antioxidant markers, markers of apoptosis and angiogenesis have been investigated. Although all these markers have been studied clinically in breast cancer none have been found useful for screening or diagnosis of breast cancer. Serum biomarkers in breast cancer could be useful for early diagnosis, prognosis and predicting response to treatment.

Serum tumour markers as biomarkers

CEA is a 200 KDa glycoprotein, first identified by Phil Gold and Samuel Freedman in 1965. This antigen is found in foetal and colon cancer tissues and thus named as CEA. Elevated levels are found in colorectal carcinoma, pancreatic carcinoma, gastric carcinoma, lung, liver, breast and ovarian carcinomas and in smokers.[10] Elevated levels are also seen in some benign conditions like bronchitis, pancreatitis, gastritis and colitis.[11] Normalization of CEA levels post-operatively is a prognostic indicator in lung cancer. Similarly, abnormal pre and post-operative serum CEA levels are useful in post-operative surveillance and in assessing the prognosis in patients with colorectal cancer.[12] Elevated serum CEA levels preoperatively is useful in assessment of the risk of recurrence and metastasis after surgery in patients with breast cancer.[13] Literature suggests that CEA may be a useful biomarker in post-surgical follow-up of breast cancer patients for an early diagnosis and for monitoring treatment.[14] Literature also suggests that CEA levels at diagnosis are correlated with stage of the disease.[15] Hence as a prognostic tool, pre-therapeutic CEA levels may be used as a biomarker in patients with bad prognosis and those who have recurrence after therapy.[16]

CA 15-3, a soluble form of Mucin 1, cell surface associated protein (MUC-1), belongs to the mucin family. It is also known as MUC-1 and is involved in cell protection and lubrication.[11] It is a high molecular weight (300 KDa) glycoprotein. CA 15-3 is expressed on apical aspects of glandular and ductal epithelial cells including breast cells. CA15-3 is over expressed at high levels in adenocarcinoma of

breast, ovary, pancreas, lung, gastrointestinal tract, urinary bladder, prostate, and endometrium.[17] Its levels are increased in cancer epithelial cells and increase with cancer development and metastasis. MUC-1 contributes to oncogenesis by inducing tyrosine kinase signaling. It has a role in immune-surveillance by blocking access of immune cells to tumours. Hence the cancer cells are protected from immune system clearance.[18] Measurement of CA 15-3 is useful as a marker for detecting recurrences and to monitor treatment of metastatic breast cancer.[19]

CA 27-29, is also called as breast carcinoma-associated antigen. have Elevated levels of CA 27-29 levels have been reported in over eighty percent of breast cancer patients. Use of serial measurements of this marker along with other tumour markers such as CA 15-3 can be used to assess recurrences and to monitor treatment response to the cancer.

Elevated levels of CA 27-29 have also been observed in cancers at other site including colon, stomach, kidney, lung, ovary, pancreas, uterus, and liver. Non-cancerous conditions like endometriosis, first trimester pregnancy, ovarian cysts, non-cancerous breast disease, kidney disease, and liver disease are also associated with elevated levels of CA 27-29.[19] Measuring serum CA 27-29 levels may be useful for post-operative surveillance and monitoring therapy.[20]

Most of the above markers are useful in assessing response to treatment and in assessing the prognosis with little utility as early biomarkers. There is a need for other markers with better sensitivity and specificity for early diagnosis of breast cancer.

Exosomes as biomarkers

Exosomes are small vesicles derived from cells measuring about 40-100 nm and present in all biological fluids.[21,22] They carry molecules including lipids, proteins, messenger ribonucleic acids (mRNAs), microRNAs (miRNAs), long non-coding RNA (lncRNA) and deoxyribonucleic acid (DNA).[23] These are gaining importance as blood-based markers for cancer diagnosis since they are released more from cancer cells than normal cells and over express certain cancer biomarkers.[24] Using a microfluidic chip for immunocapture and quantification, Circulating Epithelial cell adhesion molecule (EpCAM) positive exosomes have been found to be significantly higher in patients with breast cancer when compared to healthy controls.[25] Similarly, this chip technique was also found to be helpful in molecular classification of the breast cancer patients who showed higher levels of circulating (Her-2) positive exosomes.[25] EpCAM is an epithelial surface antigen (glycoprotein) found in epithelial intercellular junctions which mediates calcium-dependent cell-cell adhesion.[26] Apart from their role in diagnosis, exosomes have also been shown to be of promise as prognostic markers.[27, 28]

Role of non-coding ribonucleic acid

Non-coding ribonucleic acid (RNA) were found during extraction of RNA from tissues or cells as a pool of small RNA molecules which were assumed to be products of RNA degradation, arising as a result of RNA extraction procedure.[29] These molecules have a role in gene expression. These small non-coding RNA molecules include small nuclear RNAs (snRNAs) and the small nucleolar RNAs (snoRNAs) which are involved in mRNA splicing and ribosomal RNA processing respectively. Other non-coding RNA involved in the silencing of gene expression are subdivided into three types; short interfering RNAs (siRNAs) which target mRNA structure, long non coding RNAs which target chromatin for epigenetic modification and the micro RNAs (miRNAs) which regulate mRNA translation.[29]

Long noncoding RNAs

Long non-coding ribonucleic acids (lncRNAs) are longer than 200 nucleotides in length and participate in biological regulation and disease occurrence.[30,31] Functions of lncRNAs include regulation of gene methylation, activation of gene transcription, conjugation with mRNAs and microRNAs to affect translation progression.[32] The expression levels of many lncRNAs have been correlated with developmental processes and disease states.[33] Based on their genomic location, these are classified into five broad categories which are the lncRNAs, natural antisense transcripts, pseudogenes, long intronic noncoding RNAs (lincRNAs) and the divergent transcripts, promoter-associated transcripts, and enhancer RNAs. LncRNAs have been studied for their role as biomarkers in cancer. Transcriptome sequencing identified a prostate cancer associated transcript 1 (PCAT-1), an unannotated lincRNA in a prostate cancer cohort and was implicated in disease progression.[34,35] Upregulation of miR-196a and home box (HOX) transcript antisense RNA (HOTAIR) represent malignancy in gastrointestinal stromal tumours.[36]

Plasma circulating long non-coding HOTAIR has been found to be a diagnostic marker of breast cancer.[37] HOTAIR was expressed at a significantly higher level in breast cancer (BC) tissues and plasma compared to controls. It exhibited good diagnostic sensitivity and specificity [reciever operator curve (ROC) curve with an area under curve (AUC) of 0.80 (sensitivity 69.2%; specificity 93.3%)], compared to CEA [AUC = 0.50; sensitivity 65.4%; specificity 50.0%)] and CA15-3 (AUC = 0.65; sensitivity 73.1%; specificity 60.0%). The diagnostic power was further enhanced on combined use of the three markers [AUC = 0.82; sensitivity 73.1%; specificity 90.0%)].[37]Transcript antisense HOX intergenic RNA (HOTAIR) is a polyadenylated RNA having 2158 nucleotides.[38] HOTAIR has been shown to promote chromatin relocalization through Polycomb-repressive complex 2 (PRC2) thereby contributing to BC. Higher expression of HOTAIR has been shown to promote cell invasion and facilitate metastasis of BC indicating a poor prognosis.[35]

Recently, a three-long noncoding RNA signature which is upregulated in triple-negative breast cancer (TNBC) has been found to help in differentiating between TNBC and non-TNBC (NTNBC). This signature comprises of antisense noncoding RNA in the inhibitor of CPK4 (INK4) locus (ANRIL), hypoxia inducible factor 1alpha antisense RNA-2 (HIF1A-AS2), and urothelial carcinoma-associated 1 (UCA1). The diagnostic performance of these markers for differentiating between patients with TNBC and healthy individuals in terms of AUC, sensitivity and specificity were 0.830 (0.716-0.912), 0.827 (0.713-0.910), and 0.849 (0.730-0.923) for ANRIL, HIF1A-AS2, and UCA1 respectively; while their diagnostic performance to differentiate TNBC from NTNBC were 0.785 (0.660-0.881), 0.739 (0.610-0.844), and 0.817 (0.696-0.905) for ANRIL, HIF1A-AS2, and UCA1 respectively.[39]

MicroRNAs

MicroRNAs are small non-coding RNAs about 21-25 nucleotides in length which are involved in the regulation of expression of genes involved in cell proliferation and apoptosis, development, differentiation, metabolism, immunity, stress response, aging and cell cycle control. Dysregulation in miRNA expression profile could serve as molecular signatures for identifying diseases.[40] While most of the microRNAs are present intracellularly, they have also been observed in extracellular fluids including serum, plasma, cerebrospinal fluid, breast milk, colostrums, bronchial lavage, amniotic, pleural, peritonial and seminal fluids.[41-43] The circulating miRNAs are

packaged in lipid vesicles such as microvesicles, exosomes, in combination with RNA-binding proteins or both thus protecting them from ribonuclease activity and making them more stable than RNAs.[44,45] The expression profiles of circulating miRNAs have been shown to be dysregulated in various malignant diseases.[46,47] This dysregulated pattern often termed as 'miRNA signatures' can be used to discriminate healthy controls from malignant patient samples thus serving as diagnostic markers.[48] miR-125b, miR-145, miR-21, and miR-155 have been shown to be up-regulated in breast cancer patients. Data from The Cancer Genome Atlas (TCGA) involving 1110 samples identified a nine miRNA signature profile which could diagnose breast cancer with great accuracy. Of these, seven miRNAs (hsamiR21, hsamiR96, hsamiR183, hsamiR 182, hsamiR141, hsamiR200a and hsamiR429 were found to be upregulated and two miRNAs (hsamiR139 and hsamiR 145) were found to be downregulated. ROC curve analysis for the combination of these nine miRNAs showed a high diagnostic accuracy [AUC of 0.995 (95% CI, 0.988 0.999)] corresponding to a diagnostic sensitivity of 98.7% and specificity of 98.9%. A study based on TCGA and Bioinformatics data found miR-101-2, a target gene of miR-101-3p to be of diagnostic importance. The data set included 781 patients with BC and 87 adjacent noncancerous breast tissues. The diagnostic performance in terms of AUC was 0.63 (95% CI: 0.58–0.68), with a 83.9% sensitivity and 44.8% specificity. Expression of miR-101-2 was found to be significantly associat-ed with tumour (T), lymph node (N), and metastasis (M) stages of breast cancer.[49]

Diabetes mellitus has been implicated in the development of various cancers including pancreatic cancer, liver cancer, cancer of oesophagus, endometrial cancer, colon cancer, and breast cancer.[50-53] Expression of miR-124a has been reported to be down-regulated while expression of miR-30d was up-regulated in breast cancer patients with type 2 diabetes mellitus (T2DM).[54] A positive correlation was observed between miR-124a expression and high-density lipoprotein cholesterol (HDL-C), while it was negative correlation was observed with age, glycosylated haemoglobin (HbA1c), low-density lipoprotein cholesterol (LDL-C) and Estradiol (E2). On the other hand, miR-30d expression correlated negatively with HDL-C but positively with age, HbA1c, LDL-C and E2. Both miR-124a and miR-30d correlated with clinicopathological features of breast cancer patients in these patients. These markers could thus be useful for early diagnosis of breast cancer in patients with T2DM.[54]

Epithelial markers

Epithelial-to-mesenchymal transition (EMT) is characterized by multiple molecular changes which ultimately cause the epithelial phenotype to change to a mesenchymal phenotype. EMT is associated with disruption of the tight junctions between cells and a loss of cell to cell contact. These alterations result in increased invasive and metastatic capabilities of the cancer cells.[55] The regulatory pathways which control EMT also regulate cell adhesion molecules and their signaling pathways. These are important determinants of tumour cell invasion and tumour metastasis.[56] The EMT process can be studied using biomarkers which characterize the change from an epithelial to a mesenchymal phenotype. Some of these markers include markers of "cadherin switch" "epithelial (E)-cadherin Neural (N) cadherin" phenotype)[57] i.e., loss of E-cadherin; an epithelial cell adhesion protein and gain of N-cadherin, vimentin and fibronectin which are mesenchyme associated proteins. Other markers include epidermal growth factor receptor (EGFR); amember of the tyrosine

Table 1: Biomarkers in breast cancer: their advantages and disadvantages

Name of biomarker	Advantages	Disadvantages
CEA	A prognostic tool, pre-therapeutic CEA levels may be used as a biomarker in patients with bad prognosis and those who have recurrence after therapy[16]	Low sensitivity[82]
CA-15-3	One of the best investigated serum-based prognostic biomarker[83]	Low sensitivity expressed in normal cells and haematological tumours. Levels of detection 42% in BC, but approximately 59% in non-breast tumours[82,84]
CA27-29	Useful for postoperative surveillance and monitoring therapy[20]	Lack of sensitivity for early-stage disease combined with a lack of specificity[85]
Mammoglobin	MAG detects breast cancer metastasis with high specificity. Detection in peripheral blood and/ or its overexpression in breast tissues is associated with a better differentiation, a higher hormone dependence and a lower proliferation, all of which together define a better prognosis[86]	80%-90% expression in breast tumours, with a 97% sensitivity in detecting residual disease[82]
Exosomes	Promising biomarker for cancer screening, diagnosis and prognosis because they are easily accessible and capable of representing their parental cells[87]	Not cost effective
EGFR	Useful as marker for EMT in breast cancers[58]	Low sensitivity and are expressed in normal cells and other tumours[82]
Micro RNA	An early diagnostic marker with high sensitivity and specificity. It may facilitate accurate tumour stratification, predict response to treatments, predict the risk for disease recurrence or progression, or even represent novel[88]	Not cost effective
Cytokeratins	Useful as marker for EMT in breast cancers[58]	Low sensitivity and are expressed in normal cells and other tumours[82]
Circulating nucleic acids	High sensitivity and specificity[73]	Low frequency of some mutations occurring in tumours which interferes with wild-type sequences[74]

CEA = carcino embryonic antigen; CA = carbohydrate antigen; EGFR = epidermal growth factor receptor; RNA = rebonucleic acid; EMT = epithelial mesenchymal transition

kinase family, platelet-derived growth factor (PDGF) D; an important regulator of cell proliferation and nuclear factor κB (NF-κB); a transcription factor. All these play a role in breast cancer progression and their expression levels predict aggressiveness of the tumour. [58]

Other epithelial markers include expression of cytokeratins; representing an epithelial phenotype while snail, snug and twist representing a mesenchymal phenotype.[57] However, these are less specific for tumour cells.[58]

Mammoglobin

Mammaglobin (MAG) is one of the recent markers under study. Human mammoglobin (hMAM) is a product of mammoglobin (MAM) gene located on chromosome 11q12-13 is expressed at basal levels under normal conditions by the breast tissue. Its expression is increased in breast cancer.[59]The product of this gene is a glycoprotein belonging to the uteroglobin family.[60] the reported expression of MAG in breast cancer ranges from 20% - 95%.[61] Detection of MAG m-RNA in peripheral blood or its protein expression in breast tissue is more in tumours with low proliferative activity inhibitor constants (Ki-67 d”20). Circulating levels of MAG m-RNA has been shown to be specific for breast cancer with a specificity of 100% specificity of the marker.[62] Human mammaglobins A and B are homologues and members of a large family, have been reported as potentially valuable in breast cancer diagnosis and prognosis.[63] A recent report[64] stated that upregulation to induce over-expression of human mammaglobin (h-MAG in breast cancer cells can reduce the metastatic potential of breast cancer cells.[64]

MCT1, MCT4 and CD147 Genes

Tumour cells are able to spread throughout the whole body through invasive mechanisms and metastasis. Monocarboxylate transporters (MCTs) belong to a group of membrane protein family which maintain a normal to alkaline intracellular potential of hydrogin (pH) of the tumour cells despite a low extracellular pH.[65] MCTs participate in the metabolism of all cell types, but under hypoxic or ischemic conditions, tissues become dependent on MCT pathway to obtain energy. The MCTs family solute carrier (SLC16A) is composed of 14 members. These membrane proteins transport short-chain monocarboxylates (lactate, pyruvate and ketone) across the cell membrane.[66] Twelve transmembrane domains are found in MCT proteins. Among MCTs members, only MCT1-4 transport monocarboxylates couple with a proton across a cell membrane. MCT1 is the most widely expressed and is regulated by its association with the glycoprotein cluster of differentiation (CD) 147. MCT1, MCT4 and CD147 are expressed by leukocytes under normal physiological conditions. A study detected the expression of these markers in peripheral blood samples of both breast cancer patients at diagnosis and in healthy women.[67] The studied markers were more strongly expressed in patients with cancer once they are positively modulated by tumour hypoxic conditions. The increase in gene expression in the peripheral blood of the patients may occur due to the presence of circulating tumour cells (CTCs). MCT1 and CD147 markers that have shown statistical significance expression in blood samples could be used as a diagnosis marker of breast cancer.[67] The increased expression of these markers in patients with evaluated progression at diagnosis reflects an adaptation of the tumour to the acidosis caused by the activation of the glycolytic pathway and lactate production, and this adaptation prevents the activation of the apoptotic pathway in these patients.[68]

Circulating nucleic acid

Mandel and Metais[69] were the first to describe the presence of circulating, cell-free nucleic

acids (cfNAs) in the blood in 1948. These are released by tumour cells during the process of tumour development. Apoptotic and necrotic cell death results in high levels of circulating DNA, mRNA and microRNA in the blood of patients. Different concentration of cfNAs have been reported in patients with breast cancer.[70] However, the results from serum and plasma in breast cancer patients have shown contrasting findings with no difference being observed in serum levels of breast cancer patients and healthy controls.[71] Plasma levels have been reported to be significantly higher in breast cancer patients compared to benign cases.[72] Though these have good sensitivity and specificity[73] the occurrence of some mutations at a lower frequency in tumours interferes with the wild-type sequences during analysis.[74]

Adipocytokines

Adipose tissue, the largest endocrine organ secretes a wide range of adipocytokines like adiponectin, leptin, tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) which are involved in homeostasis of glucose, lipids and systemic inflammation. Adiponectin (APN) modulates glucose and fatty acid metabolism. Decreased concentrations of plasma APN are linked to obesity, insulin resistance, T2DM and atherosclerosis.[75] Obesity is the risk factor for the development of breast cancer. Lower serum APN levels have been reported in obese individuals.[76] Circulating plasma concentrations of APN are inversely related to increased risks of malignancy. Studies have found that lower circulating APN levels are associated with an increased risk of breast cancer development in post-menopausal women.[77] Others have found that decreased adiponectin levels are associated regardless of menopausal status.[78] Several studies indicates that circulating APN levels are inversely associated with risk of obesity related malignancies like breast[79] endometrial[80] and prostate cancer.[81] Tumour cells may express receptors for APN and it exerts the host protective response through cellular signaling. These factors suggest that serum adiponectin and downstream signaling targets of adiponectin can serve as a potential diagnostic marker for breast cancer.

Table 1[16,20,58,73,74,82-88] summarizes the advantages and disadvantages of the current and novel biomarkers studied in breast cancer. Among the available markers, mammoglobin and microRNAs seem to hold promise. These could find a place in clinical practise with better cost-effective methods if made available.

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