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Review

The Significance of Proteomic Biomarkers in Male Breast Cancer

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Abstract. *Breast cancer in men (MBC) is an uncommon malignancy and accounts for only 1% of all diagnosed breast cancers. By using genomic and transcriptomic approaches, researchers have been able to expand our insight into the genetic basis of breast cancer, by providing new biomarkers. We currently know that gene analysis by itself does not show the complete picture. Along with the genomic approach, proteomics are crucial for the improvement of breast cancer diagnosis, sub-classification, for predicting response to different treatment modalities and for predicting prognosis. There are great challenges in identifying discriminatory proteins and the use of specific techniques along with additional analytical tools is required. A number of techniques allow testing for proteins produced during specific diseases. In this review, an effort is made to summarize the studies and results linked to the implementation of proteomics in the field of MBC detection and diagnosis.*

Breast cancer in men (MBC) is an uncommon malignancy, it accounts for only 1% of all diagnosed breast cancers (1). According to the American Cancer Society, approximately 2,350 men will be diagnosed with breast cancer, whilst it is estimated that nearly 440 men will die from the disease in the United States in 2015. In Athens, the capital city of Greece, it has been estimated that there are approximately 20-25 new cases per year (in a male population of around 2 million) (2).

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Lifetime risk of a male developing breast cancer remains as low as 0.01% (3). The low incidence of breast malignant neoplasms in men may be due to a variety of reasons; it is unlikely for cancer to develop in vestigial parts such as male breasts (4), whereas there is not a continuous endocrine stimulation of the male breast by ovarian hormones (estrogens) (5).

The main risk factors for the development of MBC are aging, high estrogen levels, breast cancer (*BRCA*) gene mutations, *etc*. Aging is an important risk factor; the average age of first diagnosis in men is 67 years *vs*. 62 years in women (6). Moreover, high levels of estrogen have been implicated as a significant risk factor; of note, the imbalance of androgenic *vs*. estrogenic levels may be caused by a host of factors such as Klinefelter syndrome (7), high body mass index (8), lack of exercise (9), previous liver disease (10), excessive alcohol consumption (11), diabetes (12), infertility (12) and use of exogenous androgens (13), or estrogens (14). Special attention should be paid to the important role for endogenous estradiol in the etiology of male breast cancer, similarly to breast cancer in females, as was recently published by Brinton *et al*. in a large-scale collaborative study (15). Additionally, environmental issues such as exposure to ionizing radiation have been associated with higher risk of MBC (16-18). Finally, a genetic predisposition may also be considered in the etiology of MBC. More specifically, it has been shown that approximately 10% of patients with MBC carry *BRCA2* mutations and there is a high relative risk of breast cancer in men carrying these mutations (19). Depletion of BRCA1 protein is associated with increased cell proliferation in MBC similarly to breast cancer in females, although it has been reported that germline *BRCA1* mutations are less frequent in men (20). Meijers-Heijboer *et al*. have indicated that the *CHEK2* 1100delC variant gives a 10-fold risk of male breast cancer independently of *BRCA1* and *BRCA2* (21). Mutations in the *TP53* gene (22), the androgen receptor gene, *PTEN* tumor-suppressor gene, and mismatch repair genes

(*e.g. hMLH1*) have also been reported in MBC (23, 24), but none of them has been unequivocally associated with an increased risk. It must be kept in mind that mutations in these genes are uncommon in the general population and that it is possible that much of the genetic involvement in breast cancer risk may be due to co-inheritance of several low-risk common variants (25).

As far as the etiology of breast cancer is concerned, it has been well established that cancer arises from successive genetic changes which affect a number of cellular processes, such as apoptosis, angiogenesis, proliferation, growth control and metastasis (26, 27). Gast *et al*. highlighted that research was guided toward identification of markers (28). By using genomic and transcriptomic approaches, researchers were able to expand our insight into the genetic basis of cancer, by providing new biomarkers (29-31). We currently know that gene analysis in itself does not provide the complete picture. The genomics approach has its limitations. According to Chae *et al*. genomics "do not capture post-translational modification that affects protein function and stability"(32). The same authors also state that "proteins are the ultimate effector molecule of cellular functions, not genes or messenger RNAs". Therefore, the proteome, rather than the genome, is now considered to provide a more accurate reflection of both the genetic background of the cell, as well as its control over the immediate environment (33). Bearing in mind that proteomic analysis may provide the association between gene sequence and cellular physiology (34), we anticipate proteomics actually to complement gene analyses in its use in the prognosis, and evaluation of disease and its response to treatment (35).

In this review, we summarize studies and results linked to the implementation of proteomics in the field of MBC detection and diagnosis.

Proteomics

The ultimate aim of proteomics is the characterization of the information cascade *via* protein networks. This information may be the cause, or the consequence, of a disease's development (36). With the term "clinical proteomics", we refer to a sub-category of proteomics that deals with the application of proteomic technologies to clinical samples, such as blood, in order to identify unique biomarkers and biosignatures. According to Huijbers *et al*. "a biomarker, or biological marker, is a biomolecule that can be used as an indicator of a disease, based on abnormal presence, absence or changes in genes, RNA, proteins or metabolites" (37). Such proteomic technologies have application in cancer; cancer is characterized by multiple dysregulated proteins and cellular pathways involved in the onset and progression of the disease (38).

Cancer biomarkers may be divided into three main categories, with diagnostic, prognostic, and potentially predictive applications (39). Tissue, serum, plasma, cerebrospinal fluid, urine, saliva, ascites, nipple fluid, pleural fluid, or any other body fluids can be used as a matrix for the classification of breast cancer, the prediction of its response to therapy (*e.g.* targeted treatments, hormonal therapy) and for predicting prognosis by the discovery of proteomic biomarkers (32). Circulating biomarkers, such as serum, are ideal for less-invasive diagnostic procedures; the blood compartment, endowed with a protein-rich information archive (40), allows for multiple easy sample collection, thereby enhancing the clinical value of possible biomarkers (41, 42). Additionally, the proteomics approach may be able to identify protein–protein interactions, individual proteins or even driver pathways, leading towards identification of biomarker-based and personalized clinical trials in an effort to increase therapy success rate. With the development of novel targeted therapies in breast cancer, there is an increasing need for development of predictive proteomic biomarkers; in positive clinical trials, good responders may be identified with the application of proteomic techniques.

After significant efforts made in the field of clinical proteomics to discover novel breast cancer biomarkers in women, there is a small number of markers that has made it to clinical practice: uPA/PAI-1, circulating tumor cells, estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), cancer antigen (CA)15-3 and CA27.29 (43). On the other hand, data on male breast protein alterations in breast carcinogenesis are scarce due to the rarity of the disease. Data on proteomics in women should not be extrapolated to men due to the inherent discrepancies between the two (44-48).

Unfortunately, there are great challenges in identifying discriminatory proteins and the use of specific techniques along with additional analytical tools is required. A number of techniques allow testing for proteins produced during specific diseases. Proteomic platforms are divided into antibody- and non antibody-based (32). Antibody-based techniques include enzyme-linked immunosorbent assay (ELISA), western blotting (protein immunoblot), immunohistochemistry (IHC), and protein microarray (chip). Non antibody-based approaches consist of methods based on mass spectrometric (MS) technology. While the former methods require prior knowledge of the proteins that are about to be tested, as well as specific antibodies, the latter do not.

In particular, the most widely used technique for the study of proteomic biomarkers in MBC is IHC. IHC is a conventional assay with high specificity able to assess the expression of proteins. The main advantage of IHC is the fact that it provides data on cellular and spatial localization of the protein, whereas the main disadvantages of the technique are that it is semiquantative, has a low-throughput and is labor-intensive (49).

Protein microarray techniques have also been applied in a number of MBC proteomic studies. Protein microarray is a highly sensitive and high-throughput technology used for the evaluation of protein expression and interactions on multiple samples at once. In parallel, samples from more than 1,000 patients can be analyzed in a single array using a validated antibody for each protein of interest. There are distinct types of protein microarrays: analytical microarrays, functional arrays and reverse-phase protein arrays (50). In the first category, antibodies are arrayed on a surface and each array is probed with a solution that contains multiple proteins, allowing for the expression levels of a large number of proteins to be measured in parallel with a set of validated antibodies in a single array. Analytical microarrays can be used for the monitoring of differentially expressed proteins and therefore for clinical diagnostics (51). The arrays on functional protein microarrays contain either intact proteins or protein domains, and are widely used for the study of protein interactions. Lastly, in reverse-phase protein arrays a set of lysed tumor cells is immobilized onto a nitrocellulose surface and then the slide is probed with one validated antibody and later with affinity reagent. By using this technique, it becomes possible to determine the presence of altered proteins, which may indicate disease, making it an eligible platform for the discovery of new biomarkers in MBC (52).

Finally, MS has shown great potential in breast cancer studies. Protein MS is a highly sensitive analytical chemistry technique that generates a mass spectrum of proteins contained in a sample of material; the sample may be gas, solid, or liquid. MS may prove able to discover new molecular markers for early detection, prognosis and prediction of response to various chemotherapeutical agents (53-55). The principle step of MS involves ionizing chemical compounds such as intact proteins ('top-down approach') or peptides derived from enzymatically digested proteins ('bottom-up approach'). The latter approach has the advantage of providing more information per protein, since the ionization of peptides is easier than that of whole proteins (43). Subsequently, the charged molecules (proteins) or charged molecular fragments (peptides) are introduced into a mass analyze which measures their mass-to-charge ratios. Finally, the analysis of the spectra allows scientists to determine not only the protein composition of a biological sample, but also to structurally identify proteins (56). With recent advances in MS, the field of proteomics opens up new horizons for the scientific committee to discover promising tumor alterations as far as classification, prognosis and diagnosis of MBC is concerned.

Immunohistochemistry

According to IHC, breast carcinoma is divided into different subtypes; more specifically, female breast cancer is divided into luminal A and B, HER2-enriched and triple-negative subtype according to the status of ER, PR, HER2 and Ki-67 (57, 58). Of note, different treatment modalities are applied according to this categorization, *i.e.* hormonal treatment (aromatase inhibitors, tamoxifen, fulvestrant) is administered in cases of overexpression of ER and/or PR; anti-HER2 therapy (trastuzumab, pertuzumab, lapatinib, TDM1) is added to conventional therapy in tumors that overexpress HER2, *etc*. (59, 60).

Different independent research groups have tried to evaluate ER, PR, HER2 and Ki-67 status in MBC in order to subclassify the disease; these attempts in the vast majority of cases were retrospective and based on limited number of cases (Table I). ER is expressed in 75-96.9% of MBC cases amongst different published reports (61-70) and PR in 58.8-96% (61-63, 65, 67, 68); HER2 is overexpressed in 1.7-29% of MBC (61, 63, 66, 68, 71, 72). According to these data, the majority of men with breast cancer are classified as having luminal A subtype (65.7-83%) (63, 65, 73). Consequently, it is obvious that this categorization does not add much to the management of MBC and there is an unmet need for additional biomarkers in MBC. At this point, a report published by Johansson *et al*. (74) tried to sub-classify breast cancer in men in luminal M1 and M2, and suggested that these two distinct subgroups differ from the well-established intrinsic subtypes of breast cancer in women.

Consequently, there has been an effort for the evaluation of other protein markers in MBC using IHC. Androgen receptor (AR) is expressed in the majority of MBC tumors (95-81%) (68, 75), signifying the potential of anti-androgen treatment in MBC. Proteins under androgen control, such as pepsinogen C, are more highly expressed in MBC than in females with breast cancer (76). Apolipoprotein D, another androgen-induced marker, is also overexpressed in MBC (76, 77) and has been positively associated with favorable outcome in men (77). Furthermore, androgen-regulated protein prostate-specific antigen (PSA), which is used as a diagnostic marker for metastatic prostate carcinoma, is also expressed in some cases of MBC (75). Of note, connective tissue growth factor has been found to be a marker for poor prognosis; it is expressed at a high percentage in MBC and has been correlated with high proliferative index and high grade, implicating its crucial role in breast carcinogenesis (78). Additionally, a number of studies revealed a higher percentage of B-cell lymphoma 2 (BCL2) positivity in MBC (94-78.9%) (47, 66, 68, 79), implicating anti-apoptotic mechanisms in MBC (68). Furthermore, p53 immunopositivity was detected in 9-21% of patients with MBC (66-68); p53-negative tumors are more frequent in MBC than in females (80). Overexpression of p53, HER2, and c-MYC protein has been significantly correlated with poor prognosis (81). c-MYC has been detected in 20.8% of MBC cases analyzed (67). p21-Positive tumors are also significantly more frequent in MBC compared to that in females (80). Cyclin D1 is expressed in 58% of MBC cases *et*

al. and according to Rayson *et al*. PFS was decreased for MBC patients with tumors staining negatively for cyclin D (68). On the other hand, Ki67 was found positively immunostained in 38% of MBC and was correlated with a decrease in PFS (68). Serra *et al*. examined lysozyme expression in 60 MBC tissues by using IHC: 45% of the sections stained positively for this milk protein. Further analysis confirmed that nodal involvement, as well as lysozyme value, were significant predictors of short-term relapse-free survival (82). Furthermore, according to Ciocca *et al*. cytokeratins 5/6 and 14 can be potentially used for the identification of a pathologically aggressive MBC form (83). Finally, a retrospective study of 24 paraffin blocks collected from MBC patients revealed expression of both α-smooth muscle actin and CD34 in 22/24 cases, although their detection cannot be considered specific to malignancy (84).

Protein Microarray

The application of protein microarray techniques in MBC biomarker detection is limited to only a few studies. According to a collaborative large-scale biomarker analysis (44), hormone-receptor profiles were compared between breast carcinomas from 251 males and 263 females; patients were matched for age, grade and lymph node status. In this study, tissue microarrays were immunostained for ERα, -β1, -β2, and -β5, as well as PRa and -b, and AR, augmented by HER2, and CK5/6, -14, -18 and -19, to assist typing. Interestingly enough, in this study, luminal A subtype of breast cancer was the predominant phenotype in males as well as in females, whereas luminal B, basal and HER2 subtype were uncommon in MBC (Table I). Common clusters between male and female patients were revealed by hierarchical clustering, comprising total PR–PRa–PRb and ERβ1/2 clusters. Of note, the presence of ERα subunit on different clusters between the two groups was the most striking finding in this study; more specifically, in women with breast cancer, ERα clustered with PR and its isoforms, whereas in men with breast neoplasm, ERα clustered with AR and ERβ isoforms. In another study tissue microarrays were immunostained for BCL2 on 151 MBC patients (85). According to this study, BCL2 was expressed in 94% of cases; more frequently than previously described in women with breast carcinoma. Of note, in the former study, BCL2 expression was not correlated with tumor size, grade, mitotic count or overall survival. Finally, Kornegoor *et al*. conducted a study on 134 cases of MBC, by IHC of tissue microarrays for ER, PR, AR, HER2, BRST2, cyclin D1, BCL2, p53, p16, p21, Ki67, CK5/6, CK14, and epidermal growth factor receptor (86). In their study, high mitotic count and high grade were correlated with high Ki67, HER2 amplification/overexpression, p53 accumulation, high p21 expression, low PR expression, and low BCL2 expression. Furthermore, according to this study, PR negativity and p53 accumulation were correlated with decreased overall survival. Interestingly enough, in unsupervised hierarchical clustering, four groups with distinctive clinicopathological features were identified; the PR-negative/ER-positive/high-grade cluster was correlated with the poorer overall survival.

Mass Spectrometry

Regarding MBC, only a single study has been performed using MS-based technologies. Chahed *et al*. performed a thorough proteomic study of protein-expression alterations in MBC (87). Overexpression of multiple proteins in male breast tumors were identified, namely an increase in the expression of structural proteins (CK8, -18 and -19; and tropomyosin 4), glycolytic enzymes (enolase 1), stress_related proteins (peroxiredoxin 1 and peptidylprolyl isomerase A), enzymes involved in the synthesis of AMP (adenine phosphoribosyltransferase), heat-shock proteins (HSP27), galectin 1 and cathepsin D, nuclear ribonucleoproteins (hnRNP K and A2/B1), ribosomal protein S2 and proteasome β4. On the other hand, the level of tropomyosin 1 was found to be decreased in MBC tissues, insinuating a possible crucial role for this protein in MBC. Other down-regulated proteins that were identified in MBC were apolipoprotein A1 and annexin A2.

The aforementioned study was performed after the collection of breast tumor tissue sections and non-tumor tissue samples from nine patients with MBC. All patients included in the study had stage IIB at presentation and grade 2. After extraction of proteins and following solubilization, protein extracts were analyzed with 2-dimensional gel electrophoretic analysis. Specifically, both isoelectric focusing and non-equilibrium pH gradient electrophoretic analyses were performed three times. The comparison of each protein spot between healthy and tumor tissues led to the detection of proteins that were differentially expressed in MBC. Subsequently, these altered proteins were isolated; after tryptic digestion of the proteins, matrix-assisted laser desorption/ionization time of flight MS and search in protein databases were used for the identification of each protein. Western blot analyses confirmed elevated levels of CK18, tropomyosin 4, cathepsin D and HSP27 in MBC tumors. Immunohistochemical analyses of tropomyosin 4 were also performed on paraffin MBC sections and the aforementioned results were subsequently confirmed.

Conclusion

MBC is an uncommon disease with biological characteristics distinct from those of female breast cancer. However, due to the rarity of the disease and the limited published literature focusing on MBC, treatment modalities and data regarding prognosis are extrapolated from data based on women with breast cancer. Some studies have tried to provide new insights

Table I. *Current promising biomarkers for the detection of breast cancer in men (MBC).*

Investigated marker	Proteomic technique	Correlation with MBC	Reference
Estrogen receptor	Immunohistochemistry + tissue microarrays	Expressed in 75-96.9% of MBC cases	44, 61-70, 86
Progesterone receptor	Immunohistochemistry + tissue microarrays	Expressed in 58.8- 96% of MBC cases	44, 61-63, 65, 67, 68, 86
Human epidermal growth factor receptor 2	Immunohistochemistry + tissue microarrays	Overexpressed in 1.7- 29% of MBC cases	61, 63, 66, 68, 71, 72, 86
Androgen receptor	Immunohistochemistry + tissue microarrays	Expressed in 95-81% of MBC cases	44, 68, 75, 86
Pepsinogen C	Immunohistochemistry	More positive cells in MBC (76.4%) than in FBC (50%)	76
Apolipoprotein D	Immunohistochemistry	Overexpressed in MBC	76, 77
Prostate-specific antigen	Immunohistochemistry	Expressed in 23% of MBC cases	75
Connective tissue growth factor	Immunohistochemistry	Expressed in 78% of MBC cases	78
B-Cell lymphoma 2	Immunohistochemistry + tissue microarrays	Expressed in 94-78.9% of MBC cases	47, 66, 68, 79, 85, 86
p-53	Immunohistochemistry + tissue microarrays	Expressed in 9-21% of MBC patients (lower percentage than in FBC)	66-68, 80, 86
c-MYC	Immunohistochemistry	Expressed in 20.8% of MBC cases	67
Cyclin D1	Immunohistochemistry + tissue microarrays	Expressed in 58% of MBC cases	68, 86
MIB-1 (Ki67)	Immunohistochemistry	Expressed in 38% of MBC cases	68
p21	Immunohistochemistry	Expression higher in MBC vs. FBC	80
Lysozyme	Immunohistochemistry	expressed in 45% of MBC cases	82
Alpha-smooth muscle actin	Immunohistochemistry	Expressed in 91.7% of MBC cases	84
CD34	Immunohistochemistry	Expressed in 91.7% of MBC cases	84
Cytokeratin 8	2-DE and MALDI-TOF/MS	Overexpressed in MBC	87
Cytokeratin 18	2-DE and MALDI-TOF/MS + Western blot	Overexpressed in MBC	87
Cytokeratin 19	2-DE and MALDI-TOF/MS	Overexpressed in MBC	87
Tropomyosin 4	2-DE and MALDI-TOF/MS + western blot + immunohistochemistry	Overexpressed in MBC	87
Enolase 1	2-DE and MALDI-TOF/MS	Overexpressed in MBC	87
Peroxiredoxin 1	2-DE and MALDI-TOF/MS	Overexpressed in MBC	87
Peptidylprolyl isomerase A	2-DE and MALDI-TOF/MS	Overexpressed in MBC	87
Adenine phosphoribosyltransferase	2-DE and MALDI-TOF/MS	Overexpressed in MBC	87
Heat-shock protein 27	2-DE and MALDI-TOF/MS + western blot	Overexpressed in MBC	87
Galectin 1	2-DE and MALDI-TOF/MS	Overexpressed in MBC	87
Cathepsin D	2-DE and MALDI-TOF/MS + western blot	Overexpressed in MBC	87
Nuclear ribonucleoproteins K and A2/B1	2-DE and MALDI-TOF/MS	Overexpressed in MBC	87
Ribosomal protein S2	2-DE and MALDI-TOF/MS	Overexpressed in MBC	87
Proteasome beta 4	2-DE and MALDI-TOF/MS	Overexpressed in MBC	87
Tropomyosin 1	2-DE and MALDI-TOF/MS	Down-regulated in MBC	87
Apolipoprotein A1	2-DE and MALDI-TOF/MS	Down-regulated in MBC	87
Annexin A2	2-DE and MALDI-TOF/MS	Down-regulated in MBC	87

MBC: Male breast cancer; 2-DE: two-dimensional gel electrophoresis; MALDI-TOF/MS: matrix assisted laser desorption-ionization/time of flight mass spectrometry.

into the molecular mechanisms of MBC, but these findings must be verified in large-scale prospective well-designed case studies. Of note, for example MS-based technologies seem to be appealing, but unfortunately, to the best of our knowledge only one study on MBC has been published. Furthermore, the clinical application of other techniques in MBC is limited. Hence, there is an unmet need for novel well-designed, collaborative studies, focusing on MBC and evaluating protein biomarkers in this subgroup.

Conflicts of Interest

None.

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