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Comparative Proteomic and Bioinformatics Analysis of Signaling Pathways in Primary and Metastatic Breast Tumors

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Abstract

Background: Breast cancer remains a major clinical challenge due to its complexity and resistance to therapy. Understanding the molecular differences between primary and metastatic tumors is crucial for identifying novel Biomarkers and therapeutic targets.

Objectives: This study aims to investigate differentially expressed proteins between primary and metastatic breast tumors using a combined proteomic and computational approach to uncover key signaling pathways involved in disease progression.

Methods: Tissue samples from primary and metastatic breast tumors were collected and subjected to protein extraction and two-dimensional gel electrophoresis (2-DE). Differentially expressed proteins were identified and validated using public datasets (GSE218138 and GSE110810). Bioinformatic analyses, including gene ontology and KEGG pathway enrichment, were performed to interpret the functional significance of identified proteins. **Results:** Proteomic profiling revealed distinct expression patterns between primary and metastatic tumor tissues. Key proteins, including CSF1, CSF2, CCL22, and CX3CL1 were significantly upregulated in metastatic samples, while WWOX and SOCS were downregulated. These proteins were involved in cytokine signaling, TNF signaling, immune cell migration, and cytokine–cytokine receptor interaction pathways

Conclusions: Our findings suggest that the identified proteins may serve as potential biomarkers for metastatic breast cancer. Further validation in larger cohorts is necessary to assess their clinical relevance and potential as therapeutic targets.

Keywords: Breast cancer, Biomarker, Signaling pathway, Proteomics, Bioinformatics.

1. Introduction

Globally, breast cancer is the most widespread cancer among women, and constitutes a significant public health burden (1). Despite advancements in diagnostics and therapeutic modalities, breast cancer mortality remains high, particularly in advanced and metastatic stages (2). Metastatic progression remains a key obstacle in clinical oncology, as current treatment options offer restricted benefits, (4) and precise biomarkers for disease progression are still missing (5). It is estimated that around 5 to 8% of breast cancer patients are diagnosed with distant metastases at the initial presentation, and only 24–39% of those with metastatic disease survive beyond five years (3). Additionally, approximately 20% of breast cancer patients will experience a relapse, with the liver being involved in 50–70% of metastatic cases (3). Over the past decade, advances in omics technologies, especially proteomics, have become vital for unraveling cancer's complex biology (6).

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Proteomics provides a comprehensive snapshot of the protein expression landscape within cells and tissues (7). In the context of cancer, proteomic studies help uncover potential biomarkers and protein expression that can improve tumor prognosis assessment, predict treatment outcomes, classify tumor subtypes, and identify patients most likely to benefit from particular therapies (8). This study aims to identify the differential protein expression profiles and molecular signaling networks in primary versus metastatic breast tumors using a comprehensive proteomic and computational biology approach. By integrating proteomics with in silico analyses, this work seeks to identify biomarker panels associated with tumor progression, potentially offering new diagnostic and therapeutic targets.

2. Materials and Methods

All reagents and kits were used according to the manufacturer's protocols. The TRIzol® reagent for protein extraction was purchased from Invitrogen™ (Thermo Fisher Scientific, USA). The 2D electrophoresis IPG strips (pH 3–10 NL) were obtained from GE Healthcare. SDS-PAGE gel electrophoresis was performed using reagents from Sigma-Aldrich®, and silver staining was carried out with the Silver Stain Plus kit (Bio-Rad Laboratories, USA). Imaging was performed using the Bio-Rad Gel Doc™ XR+ System, and protein concentration was measured using a Nanodrop One™ (Thermo Fisher) spectrophotometer.

2.1 . Tissue Sample Collection

This study was approved by the Research Ethics Committee of Islamic Azad University, Pharmaceutical Sciences Branch, under the ethical code IR.IAU.PS.REC.1402.545. A total of 20 breast tumor tissue samples, consisting of 10 primary breast tumor specimens and 10 metastatic tumor specimens, were collected from patients who had not received any chemotherapy, radiotherapy, or hormonal therapy before surgery. Immediately after extraction, 150 mg of each tissue was immersed in liquid nitrogen and transported in cryogenic containers to the laboratory. Where they were stored at −80°C until protein extraction. Cold chain maintenance was strictly observed throughout the transport and storage process. The age range was 40–60 years. Tumors were categorized as stage II-IV based on TNM classification (primary tumor tissue and metastatic tumor of breast tissues, based on physiopathology results). Patient BMI ranged from 22 to 29 kg/m^2.

2.2. Protein Extraction

Proteins were extracted using a commercial protein extraction kit following the manufacturer's protocol. Tissues were homogenized, washed with cold PBS to remove blood contaminants, and lysed using a buffer containing protease inhibitors. The lysates were centrifuged, and the supernatant containing soluble proteins was purified using chromatography spin columns. Protein presence was confirmed through colorimetric detection and validated by 12% SDS-PAGE electrophoresis.

2.3. Two-Dimensional Gel Electrophoresis (2-DE)

Protein separation was achieved solely through two-dimensional electrophoresis (2-DE), inolving isoelectric focusing (IEF) followed by SDS-PAGE. For the first dimension, IEF was performed using 17 cm IPG strips with a pH range of 3–10. Strips were rehydrated for 16 hours with solubilized protein samples. The second dimension involved SDS-PAGE using 12% polyacrylamide gels, which were run under constant voltage until protein separation was complete.

2.4.. Gel Staining and Imaging

After electrophoresis, gels were fixed in methanol-acetic acid and stained using a silver nitrate protocol optimized for high sensitivity detection.

2.5. Image Analysis and Quantification

Protein spot detection, alignment, and quantification were conducted using the Progenesis Same Spots software.

2.6. Bioinformatics and Dataset Integration

To complement the proteomic analysis, publicly available gene expression datasets, GSE218138 for primary breast tumors and GSE110810 for metastatic breast tumors were retrieved from the Gene Expression Omnibus (GEO). Data normalization, annotation, and integration were performed using R software version 4.5.0.

2.7. Pathway and Enrichment Analysis

Proteins with statistically significant differential expression were subjected to Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. These analyses were performed utilizing the Enrichr database platform.

3. Results

3.1. Protein Profiling via 2-DE

2-DE revealed clear differences in protein expression profiles between primary and metastatic breast tumor samples, with several protein spots showing significant differential expression. Notably, six proteins demonstrated substantial changes between the two groups. Among these, CSF1, CSF2, CX3CL1, and CCL22 were found to be upregulated in metastatic tissues. In contrast, WWOX and SOCS were downregulated in metastatic samples (Figure 1 and Table 1).

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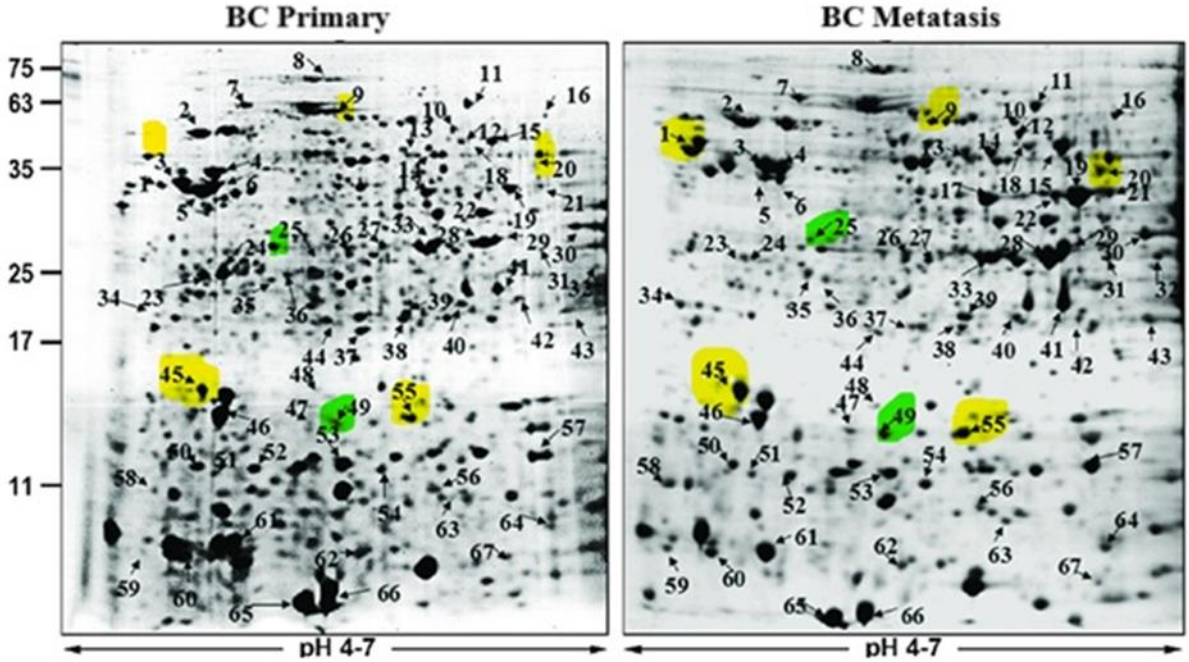

Figure 1. Differentially expressed protein between primary and metastatic breast cancer tissue observed using 2DE.

Table 1. Differentially expressed proteins between primary and metastatic breast cancer tissues.

Protein No.	Protein Name	Isoelectric pH	Protein Expression
1	CSF1	6.5	Upregulated
20	CX3CL1	6.9	Upregulated
45	CSF2	6.7	Upregulated
55	CCL22	5.5	Upregulated
25	WWOX	6	Downregulated
49	SOCS	5.9	Downregulated

3.2. Box Plot Visualization

Box plot analysis illustrated a comparable range of gene expression levels across both groups, with median values and interquartile ranges displayed (Figure 2). Outliers are indicated as individual dots beyond the whiskers.

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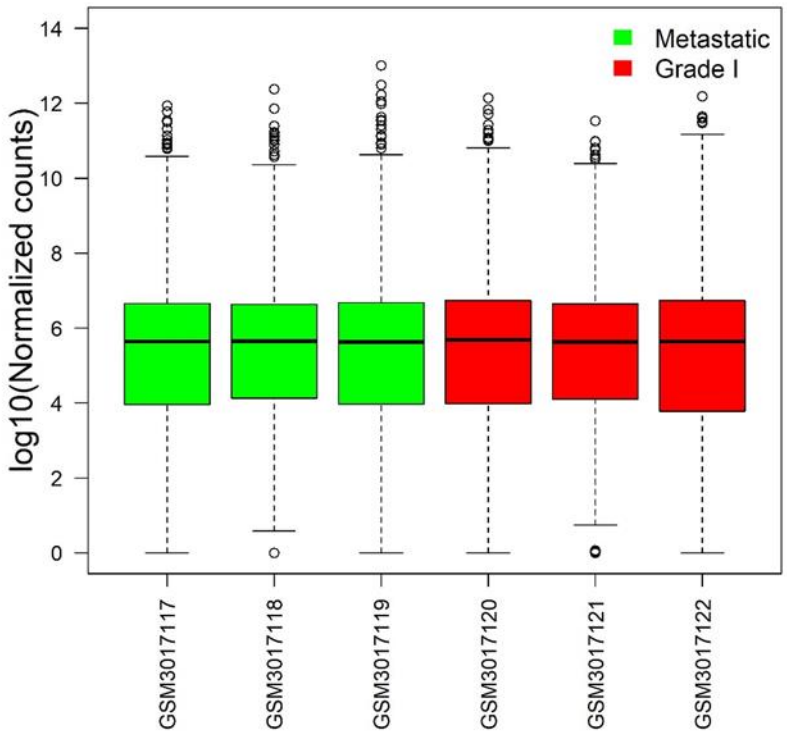

Figure 2. Boxplot of normalized gene expression counts across breast cancer tissue samples.

3.3. Gene Ontology and Pathway Enrichment

GO and KEGG pathway enrichment analyses were carried out using the CSF1, CX3CL1, CSF2, CCL22, WWOX, and SOCS proteins. The results revealed significant enrichment in immune and inflammatory signaling pathways, including the TNF signaling pathway and cytokine-cytokine receptor interaction, suggesting a key role of cytokine signaling in metastatic transformation. Additionally, GO biological processes such as "cellular response to cytokine stimulus" and "positive regulation of microglial and glial cell migration" were highlighted, which indicate potential involvement in tumor interactions with the microenvironment and cell motility regulation.

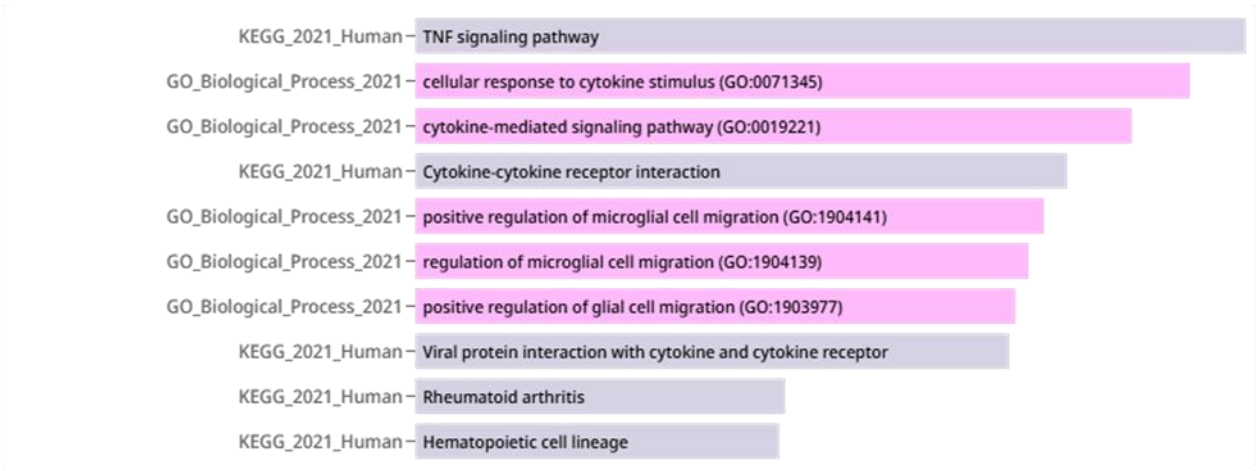

Figure 3. GO and KEGG pathway enrichment analysis of differentially expressed proteins between primary and metastatic breast tumors.

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4. Discussion

This study identified key proteomic differences between primary and metastatic breast tumor tissues, particularly involving proteins linked to immune signaling and tumor progression. The upregulation of CSF1, CSF2, CX3CL1, and CCL22 in metastatic samples suggests an enhanced pro-inflammatory and immunosuppressive microenvironment, while the downregulation of WWOX and SOCS implies the loss of tumor-suppressive and regulatory mechanisms during metastasis.

Colony-stimulating factor-1 (CSF1) and its receptor (CSF1R) have been associated with the development and advancement of breast cancer (9). We have demonstrated that CSF1 is upregulated in metastatic breast tissue, which is in line with the study by Richardsen et al., who showed that high expression of CSF1 and CSF1R and high density of tumor-associated macrophages (TAMs) and CD3+ T-lymphocytes were correlated with the progression of breast cancer (10). Other studies on breast cancer have shown that higher expression levels of CSF1 and CSF1R proteins are associated with increased inflammation and a worse prognosis (11). Another study by Aharinejad et al. reported significantly higher serum CSF1 levels in patients with early-stage breast cancer compared to those with benign tumors, further supporting our findings on CSF1 expression (12).

Colony-stimulating factor-2 (CSF2), which is also known as granulocyte-macrophage colony-stimulating factor (GM-CSF), is a cytokine that promotes tumor progression, metastatic, and the development of chemotherapy resistance (13). Our findings show that CSF2 is upregulated, which is consistent with the study by He et al., who demonstrated that CSF2 is markedly overexpressed in breast cancer cells. Additionally, CSF2 has been shown to induce inflammatory phenotypic alterations in adipocytes via the STAT3 signaling pathway, further highlighting its role in the tumor microenvironment (14). In addition, a study by Klemm demonstrated that targeting TAMs at different stages of the metastatic cascade using the CSF1R inhibitor BLZ945 in murine breast-to-brain metastasis models led to significant antitumor effects in both preventive and therapeutic preclinical trials (15). This finding is consistent with our results, which show that CSF2 is upregulated in metastatic breast tumors compared to primary breast tumors, suggesting its potential as a biomarker for targeted therapeutic strategies.

The expression of CX3CL1/CX3CR1 has been linked to various cancers, such as breast cancer (16). CX3CL1 secreted within the tumor microenvironment acts as a chemoattractant and plays a crucial role in the metastasis of cancer cells that express CX3CR1 (17). CX3CL1 in the tumor microenvironment facilitates the migration of various CX3CR1-positive tumor types, including breast cancer (18). In our study, we demonstrated that CX3CL1 is upregulated in metastatic breast tumors, which is consistent with the findings of Tsang et al., who reported elevated CX3CL1 expression in breast tumors correlated with significantly worse overall survival outcomes (19). Additionally, Dreyer et al. reported a positive prognostic association between CX3CL1 overexpression and breast cancer survival outcomes, which is in line with our findings (20). Their study also showed that overexpression of CX3CL1 attracted tumor-suppressing lymphocytes, such as natural killer (NK) cells, and significantly inhibited both tumor growth and lung metastasis in the syngeneic 4T1 breast cancer mouse model (20).

Their study also showed that overexpression of CX3CL1 attracted tumor-suppressing lymphocytes, such as natural killer (NK) cells, and significantly inhibited both tumor growth and lung metastasis in the syngeneic 4T1 breast cancer mouse model (20). Liang et al. also demonstrated that CX3CL1 may play a specific role in the metastasis of breast cancer to the spine, which aligns with our results (21).

CCL22 is a chemokine that plays a role in breast cancer by influencing immune cell recruitment, particularly regulatory T cells (Tregs), into the tumor microenvironment (22, 23). In our study, we showed that CCL22 is overexpressed in metastatic breast tissue, which is consistent with the study by Jafarzadeh et al., who observed elevated serum CCL22 levels in patients with breast cancer, with increased levels correlating to more advanced stages of the disease (24). Another study by Lecoq et al. demonstrated that vaccines targeting CCL22 can enhance anti-tumor immunity in vivo by promoting the infiltration of CD8+ T cells and M1 macrophages into the tumor microenvironment (25). This is consistent with our findings, which show that CCL22 is overexpressed and suggests its potential as a promising target biomarker for therapeutic strategies.

Recent studies have linked reduced or absent WWOX expression to tamoxifen resistance in breast cancer and have proposed that WWOX could serve as a potential predictive marker for treatment outcomes (26). We demonstrated that WWOX is downregulated in metastatic breast tissue, in line with the study by Pospiech et al., which found that elevated WWOX expression suppresses cell proliferation in suspension and reduces tumor growth in xenograft models (27). Chang et al. also showed that WWOX overexpression markedly suppressed breast cancer cell proliferation and metastasis by inhibiting STAT3 activation, which interacts with JAK2 to inhibit the phosphorylation of both JAK2 and STAT3 (28). Additionally, reduced WWOX expression has been linked to the basal-like subtype of breast cancer and lower disease-free survival rates in patients, which aligns with our findings (29).

The suppressor of the cytokine signaling (SOCS) protein family serves as an inhibitors of the cytokine-activated JAK/STAT signaling pathway (30). SOCS1 and SOCS3 are negative regulators of JAKs and are critically involved in both inflammatory processes and the development and progression of cancer (31). In our study, SOCS has been downregulated, which aligns with the findings of Lv et al., who found that reduced SOCS1 expression plays a significant role in the development of breast cancer, particularly concerning lymph node metastasis and clinical tumor staging (32).

5. Conclusion

The novelty of this study lies in its integration of computational and proteomic approaches, wherein differentially expressed proteins were identified using 2-DE, and cross-referenced with GEO datasets for both primary and metastatic breast tumors. Through comparative analysis of tumor tissues, we identified key differentially expressed proteins, including CSF1, CSF2, CCL22, CX3CL1, WWOX, and SOCS, which are closely linked to immune modulation, inflammatory signaling, and metastatic progression.

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This integrative strategy provides a system-level biomarker discovery framework that goes beyond conventional single-pathway or gene-centric approaches and introduces a novel algorithmic model with potential applications in cancer prognosis and targeted therapy.

Abbreviations

IEF, Isoelectric Focusing; GEO, Gene expression Omnibus; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, Biological Processes; MF, Molecular Functions; CC, Cellular Components; TAMs, Tumor-associated macrophages; CSF1, Colony-stimulating factor-1; CSF1R, Colony-stimulating factor-1 receptor; CSF2, Colony-stimulating factor-2; GM-CSF, Granulocyte-macrophage colony-stimulating factor; NK cells, Natural killer cells, Tregs, Regulatory T cells; SOCS, Suppressor of cytokine signaling.

Author contribution

N.Kh.: Conceptualization; Investigation; Methodology; Visualization; Resources; Writing - original draft; **F.A.:** Conceptualization; Design, Data curation; Formal analysis; Software; Validation; Investigation; Methodology; Project administration; Resources; Supervision; Writing - original draft; Writing - review & editing. M.M.: Investigation; Visualization; Writing - original draft; Writing - review & editing.

Data availability statement

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Conflict of interest

The authors declare no conflicts of interest related to the publication of this article.

Competing interest

All authors confirm they have no competing interests to declare.

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