

## Identification of Immunogenic Proteins in Early and Advanced Stages of Breast Cancer: An Immunoproteomics Study

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### Abstract

**Background:** Early detection of breast cancer (BC) is extremely important as late diagnosis has been associated with a high rate of mortality. Immunogenic proteins and autoantibodies have been considered as favorable targets for early detection and targeted therapy in cancer. Accordingly, the present study aimed to identify the immunogenic antigens in both early and advanced stages of BC via a serologic proteome analysis (SERPA) approach.

**Method:** This is a case-control study wherein we separated the proteins from BC tissues in the early stages (n = 10) and advanced stages (n = 10) utilizing two-dimensional electrophoresis (2DE) and then transferred them onto a Polyvinylidene Difluoride (PVDF) membrane. To explore the tumor antigens reacting with antibodies, two-dimensional (2D) blots of tumor tissues in the early and advanced stages were separately probed with the sera from the same patients. Afterwards, we identified antibody-reactive proteins via liquid chromatography with tandem mass spectrometry (LC-MS/MS).

**Results:** Fibrinogen beta chain (FGB), protein deglycase DJ-1(PARK7), and peroxiredoxin-2 (PRDX2) were the highly reactive antigens identified in the early-stage patients. In addition, RuvB-like1 (RUVBL1) and triose phosphate isomerase (TPI) were recognized as the immune reactive proteins in the late-stage patients.

**Conclusion:** The results herein revealed that the immune-proteome pattern of BC patients changes along with tumor progression from primary to advanced stages. Moreover, immunogenic proteins seemed to stimulate the humoral immune system to produce autoantibodies in the initiation phase of BC; these autoantibodies could be employed as complementary factors for early detection of BC. The findings are however preliminary, and further studies with a larger sample size are required for verification and validation of previous findings.

**Keywords:** Breast neoplasms, Immunoreactive, Peptides, Autoantibodies, Serologic proteomic analysis, LC-MS/MS

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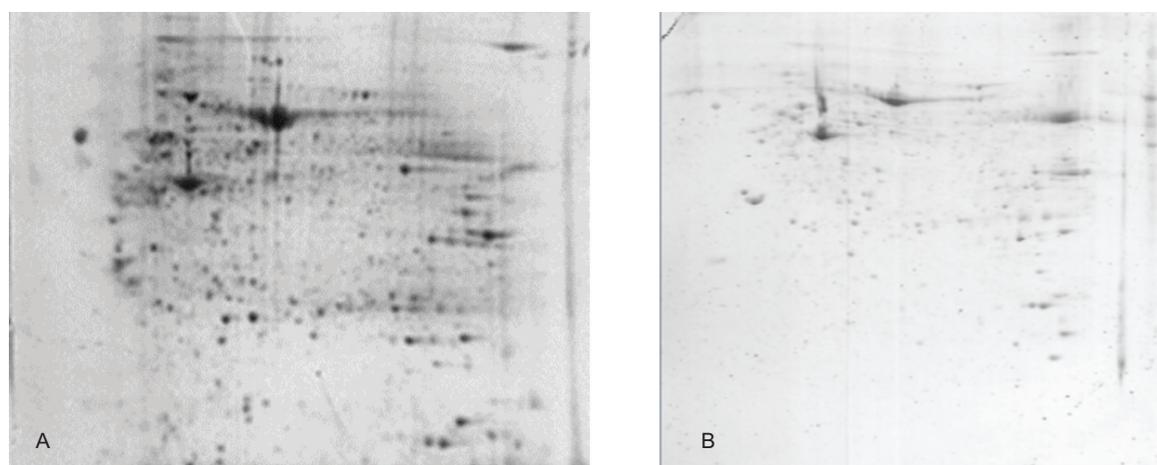
## Introduction

Breast cancer (BC), as a prevalent malignancy, accounts for 18% of all female cancers, whose occurrence around the world is changing due to inevitable changes in life-style and advances in medical diagnosis tools.<sup>1</sup> This malignancy is one of the most frequent types of cancer in Iran, which has burdened the health-care system with a high annual cost. In this regard, an epidemiologic study revealed that BC among Iranian women is diagnosed at advanced stages with a lower mean age than the global average.<sup>2</sup> Due to the lack of early symptoms in the primary phase of breast cancer, it is usually diagnosed at higher stages; therefore, the treatment options are limited for BC patients and are associated with difficulties.<sup>3</sup> Moreover, early detection of breast cancer is extremely important since women whose breast cancer is detected at early stages have a higher survival rate in the first five years.<sup>4</sup> Immunogenic proteins and their cognate autoantibodies are the favorable targets for early detection and cancer immunotherapy.

During carcinogenesis, the proteins of normal cells undergo numerous variations, such as overexpression, altered glycosylation, and post-translational modifications (PTM), all of which could provoke the immune system into autoantibody generation.<sup>5</sup> The serum levels of cancer neo antigens are scarcely discoverable at the early stages of tumor progression, while a small amount of tumor-associated antigens (TAAs)

can stimulate the immune system to produce antibodies.<sup>6</sup> The production of serum autoantibodies against TAAs has been observed in a variety of cancers, including B-cell acute lymphoblastic leukemia,<sup>7</sup> hepatocellular carcinoma,<sup>8</sup> colon cancer,<sup>9</sup> lung cancer,<sup>10</sup> prostate cancer,<sup>11</sup> breast cancer,<sup>12</sup> and primary open angle glaucoma.<sup>13</sup> Of note, the most important feature of circulating autoantibodies (AABs), compared to classic tumor markers, is their detectability prior to manifestation of clinical symptoms.<sup>14</sup> In this regard, Chapman et al. observed that AABs production against TAAs in breast carcinoma patients can be measured up to four years before tumor recognition by mammography.<sup>15</sup> Therefore, this remarkable data signifies the role of the immune system by recognizing the TAAs as “nonself” and provides a rapid humoral immune response in the early stages of BC. In the early stages of cancer, B cells, by class switching, can produce 5000–20,000 antibodies/min confronted with tumor-associated antigens.<sup>16</sup>

To date, several autoantibodies have been reported as BC biomarkers against known oncogenic proteins, such as p53, MUC1, HER2, and cyclin B1, with high degrees of diagnostic value.<sup>17</sup> In addition, a panel of autoantibodies against lesser-known immunogenic proteins has been reported in BC. These include ribosomal protein S6, eukaryotic elongation factor 2, eukaryotic stretching factor 2 kinase, heat shock protein 90 (HSP90), Ku protein, and



**Figure 1.** This figure shows the two dimensional electrophoresis (2-DE) proteome pattern of tumoral breast tissue (A) and normal breast tissue (B). The two dimensional (2D) gels were stained with Coomassie Brilliant Blue G-250.

topoisomerase I.<sup>18</sup> Immunogenic proteins on the other hand could be suitable targets for cancer diagnosis, prognosis, targeting, and vaccination.<sup>19</sup> Additionally, the autoantibodies against such proteins could be considered as potential serum biomarkers for early detection of BC.<sup>18</sup> Furthermore, serological proteome analysis (SERPA) is a high throughput method for identification of tumor antigens reacting with antibodies.<sup>20</sup> Since there is scarce information regarding immunogenic proteins in BC, the present study aimed to explore a panel of immunoreactive antigens for Iranian BC patients in both primary and advanced stages. To this end, we used 2D blots of breast tumor tissues lysates in different stages as sources of antigens and separately probed them with the sera from BC patients and healthy individuals. Immunoreactive proteins were then identified via liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis.

## Materials and Methods

### *Tissues and sera specimens*

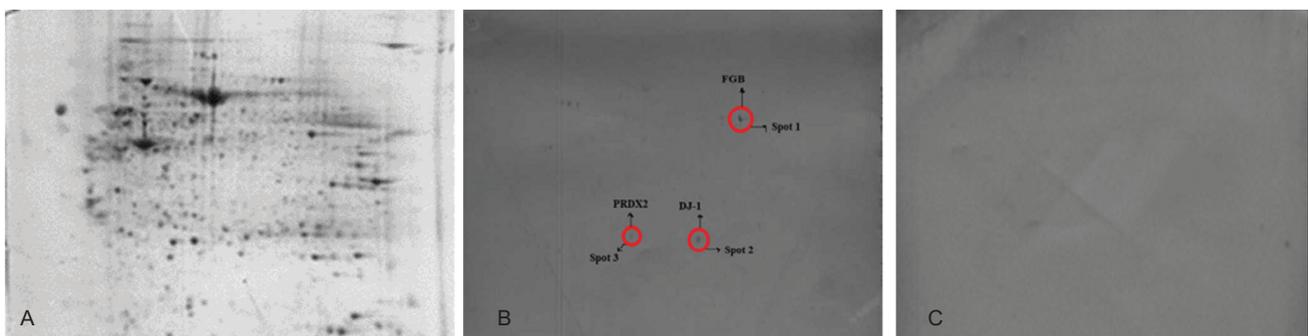
The Ethics Committees of Babol University of Medical Sciences approved this case-control study (IR.MUBABOL.HRI.REC.1398.245). We also took written consent from all the participants for blood and tissue samples collection. The tissue samples of the BC patients were collected following an operation in the Surgery Department of Shiraz Central Hospital (MRI) and Faghihi Hospital (Shiraz, Iran) over six months. All the tumor tissues were the invasive ductal carcinoma

(IDC) type, which we categorized in two groups (clinical stages I and II or early stages,  $n = 10$ ; clinical stages III or advanced stages,  $n = 10$ ). Concerning the subjects, this study included women aged between 32 and 60 years with tumor grades I, II, and III. At the time of diagnosis, the patients had not experienced distant metastasis. Moreover, normal breast tissues were taken from healthy women after cosmetic surgery ( $n = 5$ ).

The first sera was collected from 20 patients with confirmed BC (female, median age of 32-60 years, early stages,  $n = 10$ ; advanced stages,  $n = 10$ ) prior to the surgery or any interventional treatments. A second set of sera was taken from healthy volunteers with no history of cancer or autoimmunity in themselves or their first-degree family members ( $n = 10$ , female, median age of 35-60 years). The final set of sera was collected from the patients with autoimmune diseases (confirmed systemic lupus erythematosus (SLE),  $n = 2$ ). Both sera and tissue samples were then stored at  $-80^{\circ}\text{C}$  until use. Table 1 represents the clinicopathological characterization of the 20 newly diagnosed BC patients.

### *Lysates preparation*

Primarily, we washed the tissue specimens with 1 ml cold phosphate-buffered saline (PBS) to remove the blood and necrotic tissues and then stored them in liquid nitrogen. The tissue samples (0.1 g) were then placed into a mortar and turned into powder using liquid nitrogen and a mortar handle. Afterwards, the samples were homogenized on ice in 1 ml of lysis buffer (7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)



**Figure 2.** This figure represents the two dimensional (2D) gels and blots obtained from the tumor tissues lysates at an early stage; the gel stained with Coomassie Blue G-250 (A); the tumor tissue blots probed with the patients' sera at an early stage (B); the tumor tissue blots probed with the sera from the healthy controls (C). The blots were visualized by 3, 3'-diaminobenzidine (DAB) reagent. The spot number and identified proteins are related to table 2.

**Table 1.** Clinicopathological characterization of the patients with BC

Patient	Age	Tumor type	TNM stage	LVI	Tumor grade
P1	32	IDC	IA	negative	II
P2	42	IDC	IIA	positive	II
P3	45	IDC	IB	positive	II
P4	50	IDC	IIB	positive	I
P5	60	IDC	IA	negative	I
P6	48	IDC	IB	negative	II
P7	59	IDC	IIA	negative	III
P8	36	IDC	IIB	positive	III
P9	39	IDC	IIA	positive	II
P10	60	DC	IA	negative	I
P11	54	IDC	III B	positive	II
P12	48	IDC	III C	positive	II
P13	46	IDC	IIIA	positive	II
P14	39	IDC	III C	positive	II
P15	33	IDC	III B	positive	II
P16	47	IDC	III C	positive	II
P17	50	IDC	III A	positive	II
P18	60	IDC	III B	positive	II
P19	52	IDC	III C	positive	II
P20	59	IDC	IIIA	positive	II

LVI: lymphovascular invasion; TNM: Tumor, node, and metastasis; BC: Breast cancer; IDC: Invasive ductal carcinoma

dimethylammonio]-1-propanesulfonate (CHAPS), 2% immobilized pH gradient (IPG) buffer, 40mM dithiothreitol (DTT) in the presence of protease inhibitors. Homogenates were centrifuged at  $14,000 \times g$  for 15 min at  $4^{\circ}\text{C}$ . Subsequently, we collected the supernatant and determined the total protein concentration in each sample utilizing the Bradford method and aliquoted into 500  $\mu\text{l}$ ; they were kept at  $-80^{\circ}\text{C}$ . All the components were purchased from GE Healthcare (Sweden).

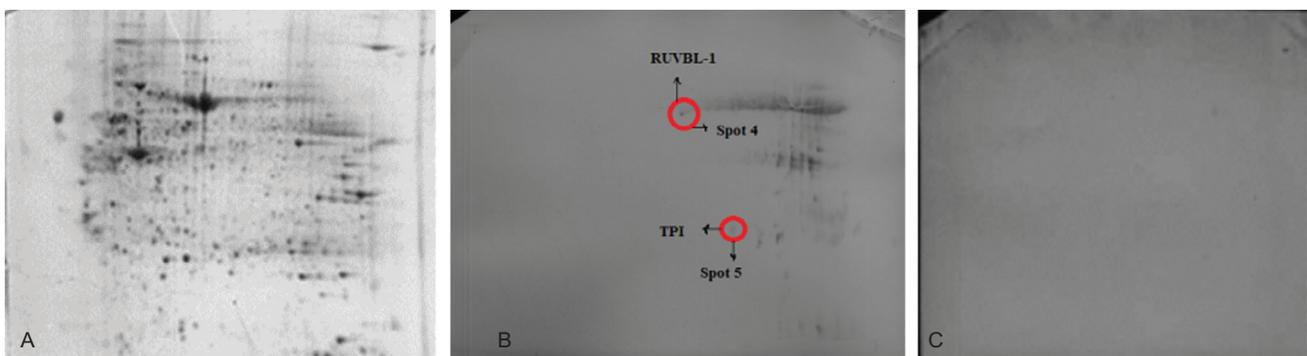
#### Two-dimensional gel electrophoresis (2-DE)

2-DE is a combination of isoelectric focusing (IEF) and Sodium Dodecyl Sulfate Polyacrylamide

Gel Electrophoresis (SDS-PAGE), in which proteins are separated based on isoelectric point (PI) and molecular weight.<sup>24</sup>

#### 2D western blot analysis

For 2D western blotting, we transferred the proteins from the 2D gels onto PVDF membranes with a semi-dry blotter (BioRad Laboratories, Hercules, CA, USA) under a voltage constant condition (22v) for 1 h. Thereafter, the PVDF membranes were blocked with 5% skimmed milk in PBS/Tween for 16 hours at room temperature. After blocking, the PVDF membrane was incubated with sera as a source of antibodies with



**Figure 3.** This figure represents the two dimensional (2D) gels and blots obtained from the tumor tissues lysates at a late stage; the gel stained with Coomassie Blue G-250 (A); the tumor tissue blots probed with the patients' sera at a late stage (B); the tumor tissue blots probed with the sera from the healthy controls (C). The blots were visualized by 3, 3'-diaminobenzidine (DAB) reagent. The spot number and identified proteins are related to table 2.

a dilution of 1:50 in blocking buffer with 2 h of incubation time. Subsequent to washing the PVDF membrane three times with PBS and 0.05% Tween-20 (PBST), we incubated it with goat anti-human IgG-HRP, as secondary antibody, with a dilution of 1:1000 in blocking buffer for 1 hour at RT on a shaker. The membrane was washed again three times with PBST and once with PBS.<sup>21</sup> The positive spots were then detected with 3,3'-Diaminobenzidine (DAB). We dissolved 0.05 gr DAB in 50 ml TBS followed by adding 72  $\mu$ l H<sub>2</sub>O<sub>2</sub>. We then spilled it on the surface of the PVDF membrane. After 15 min, brown dots appeared at the location of the antigen-antibody reaction.

#### Spot selection

The PVDF membranes were scanned with a resolution of 300 dpi via a densitometer scanner (Bio-Rad Laboratories) and recorded in TIFF format. To map the spots with different immunoreactivities, we analyzed the blots using Prodigy software (version 1.0, Nonlinear Dynamics, Newcastle, UK). In addition, the matching process was confirmed by eye in at least three images.

#### Protein identification via LC - MS/MS

The protein spots reacting with cancer serum antibodies were manually cut from the 2D gels stained with Coomassie Brilliant Blue G-250. We rehydrated the gel pieces in a Trypsin/LysC solution. Digestion was carried out overnight at 37°C. Moreover, we purified the peptides via reversed phase extraction and analyzed them with LC-MS/MS. Statistical confidence limits of 95%

were applied for the proteins. Cov (%) >53 were considered to indicate the statistically significant differences ( $P < 0.05$ ). Furthermore, mass selection of the analyte with mass-to-charge ratio (m/z) was followed by fragmentation and analysis of the fragments. We then analyzed the produced tandem mass spectral data with the Protein Pilot software.

#### Protein-protein interactions and functional analysis

To survey the relationship between the identified proteins, protein-protein interactions (PPI) were analyzed via the STRING database (<http://string-db.org>). Moreover, the biological roles of the recognized proteins were interpreted by the GeneCards database (<https://www.genecards.org>) and the KEGG PATHWAY database (<https://www.genome.jp/kegg/pathway.html>).

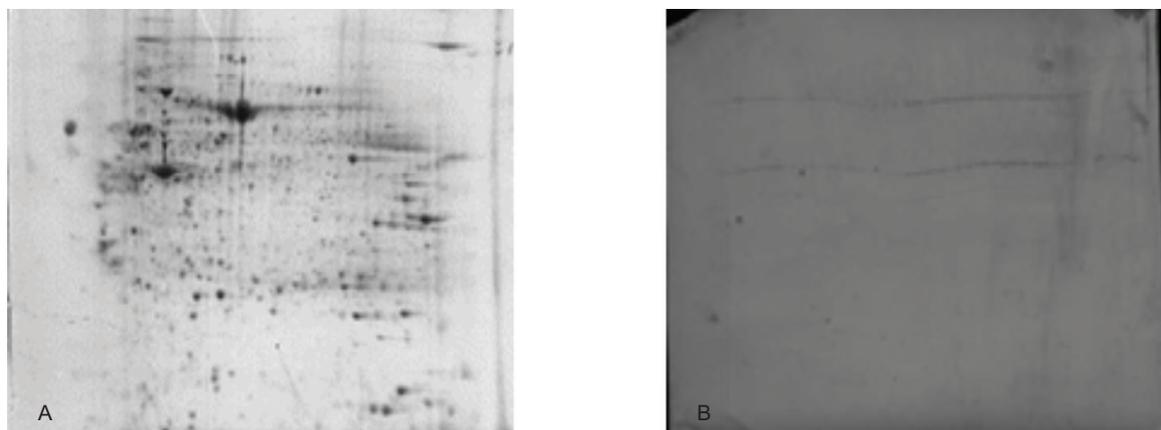
## Results

#### Bradford assay

We calculated the total protein concentration using Bradford assay; for the primary, advanced, and normal groups, 4.3  $\mu$ g / $\mu$ l, 6  $\mu$ g / $\mu$ l and 5.5  $\mu$ g / $\mu$ l were obtained, respectively.

#### 2-DE proteome pattern

As shown in figure 1, despite overall similarities, the proteins' expression patterns indicated some differences between the tumoral and normal breast tissues. Figure 1 depicts the extracted proteins separated with 2-DE in the tumoral and normal breast tissues.



**Figure 4.** This figure represents the two dimensional (2D) gels and blots of the tumor tissues lysates at a late stage and sera from SLE patient; the gel stained with Coomassie Brilliant Blue G-250 (A); the tumor tissue blots probed with the sera from the SLE patient (B). SLE: Systemic lupus erythematosus

**Table 2.** Description of the identified proteins via mass spectrometry

Spot number	Protein name	Accession No.	COV (%)	Peptide(%)	pI	MW(kDa)
1	Fibrinogen beta chain	sp P02675	56	83	8.3	55.9
2	Protein deglycase	sp Q99497	87	38	6.7	19.8
3	Peroxiredoxin-2	sp P32119	75	54	5.7	21.8
4	RuvB-like 1	sp Q9Y265	87	30	6.02	50
5	Triosephosphate isomerase	sp P60174	75	69	6.45	26

MW: Molecular weight; pI: Isoelectric point; COV: Coverage; No.: Number

### *Differential immunogenic proteins*

To identify the cancer-specific immunogenic proteins in different stages of BC, we probed 2D blots of tumor tissues lysates at early stages with the sera from the BC patients at early stages. We also investigated 2D blots of tumor tissues lysates at advanced stages with the sera from the BC patients at advanced stages. To identify the tumor proteins that only reacted with patients' sera, 2D blots of tumor tissue lysates were also probed with the normal match individuals' sera. Moreover, we performed the experiment in triplicate for each group.

Our results indicated that the immunoreactive proteins of our early-stage patients were different with those in advanced stages. In this regard, our results revealed the presence of at least three reactive spots at early stages and two reactive spots at advanced stages. On the other hand, the reactive spots derived from the tumor tissues of either early stages or late stages were reproducible and had no visibility or reactivity with the healthy individuals' sera. Figures 2 and 3 illustrate the results.

Additionally, 2D blots of tumor tissue lysates

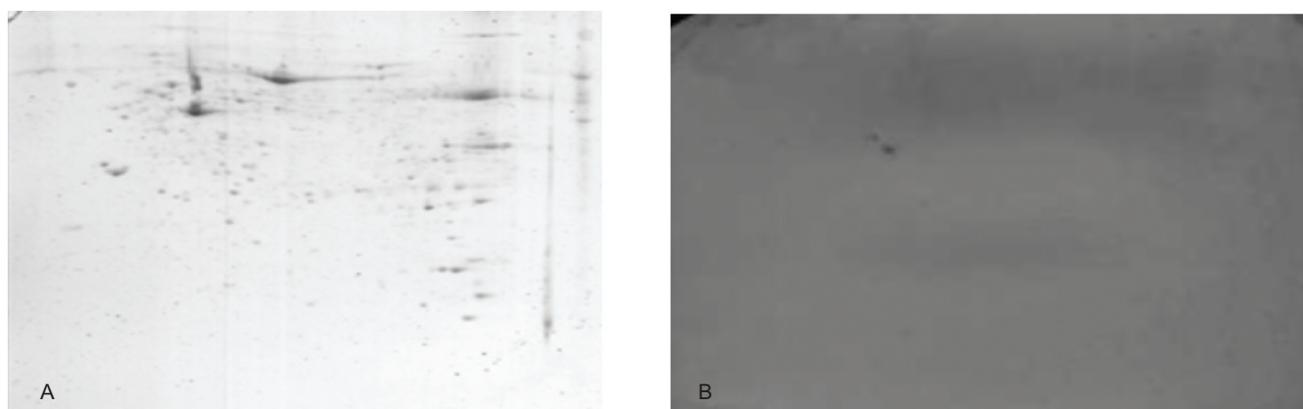
were probed with the sera from the SLE patients to further differentiate between the tumor-specific immunogens and tumor non-specific immunogens that react with ordinary autoantibodies. As shown in figure 4, we observed no visibility or reactivity with the sera of those with SLE. Finally, we investigated 2D blots of the normal tissue lysates with the healthy individuals' sera; the results showed no visibility or reactivity (Figure 5).

### *Protein identification via mass spectrometry*

The spots representing reproducibility without visibility or reactivity with the sera from healthy individuals and SLE patients were sent for mass spectrometry analysis. Table 2 demonstrates the descriptions of the identified proteins. In general, we identified five highly reactive proteins in the BC patients; fibrinogen beta chain (FGB), protein deglycase DJ-1(PARK7), and Peroxiredoxin-2 (PRDX2) were the immune reactive antigens in the early-stage patients, whereas RuvB-like 1 and Triose phosphate isomerase (TPI) were recognized as the immune reactive biomarkers in those at late stages.

### *STRING analysis*

The STRING database involves both well-



**Figure 5.** This figure represents the two dimensional (2D) gels and blots of the normal tissue lysates; the gel stained with Coomassie Brilliant Blue G-250 (A); the normal tissue blots probed with the sera from the healthy individuals (B).

known and predicted protein interactions in which genes or proteins are represented with nodes and edges marked with different colors. The functional linkages between the two proteins are ranked based on confidence score, with each score providing information about the functional similarity between the two proteins. The confidence score was previously reported as highest (0.9-1), high (0.7-0.8), medium (0.6-0.4), and low (0.1).<sup>22</sup> Figure 6 depicts the STRING analysis of the identified proteins. Based on STRING analysis, we herein calculated the confidence score of the identified proteins and their functional partners to be at 0.999, reflecting the maximum functional linkage between these proteins.

*Molecular function and biological process of the identified proteins*

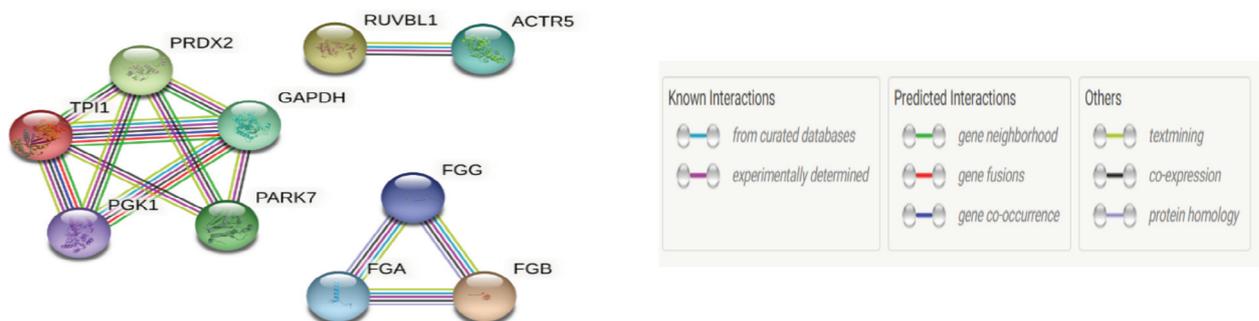
We utilized the GeneCards and KEGG PATHWAY databases for investigating the molecular function and biological process of the identified proteins. The results are summarized in table 3.

**Discussion**

In the present study, we identified a panel of immunogenic proteins in BC patients in different

stages of the disease. We also realized that the immunoproteomics pattern of the early-stage patients is different from that of the advanced-stage ones. These findings could be attributable to the changes in the expression pattern of proteins during tumor progression. In addition, the quality and severity of immune response varies from stage to stage in BC patients.<sup>23</sup>

Fibrinogen beta chain (FGB), DJ-1 (PARK7), and PRDX2 are the highly immunoreactive proteins identified with the LC- MS /MS approach in BC patients in early stages. Triosephosphate isomerase (TPI) and RUVBL1 are two of the other candidates identified for BC patients in advanced stages. As the immune system is an indispensable player during tumorigenesis and cancer development, autoantibodies, particularly cancer antigen-specific autoantibodies, may be used as early biomarkers for cancer detection and prevention. More importantly, the detection of these autoantibodies may be indicative of novel treatment strategies (development of monoclonal antibodies against the same cancer antigen to cure the disease). Functional analysis, as summarized in table 3, demonstrated that the identified proteins had functional similarities and participated in various biological processes, such



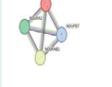
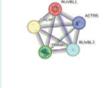
**Predicted Functional Partners:**

**score:**

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase; Has both glyceraldehyde-3-phosphate dehydrogenase and nitrosylase activities...	0.999
ACTR5	Actin-related protein 5; Proposed core component of the chromatin remodeling INO80 complex which is involved in transcrip...	0.999
FGA	Fibrinogen alpha chain; Cleaved by the protease thrombin to yield monomers which, together with fibrinogen beta (FGB) and ...	0.999
FGG	Fibrinogen gamma chain; Together with fibrinogen alpha (FGA) and fibrinogen beta (FGB), polymerizes to form an insoluble fi...	0.999
PGK1	Phosphoglycerate kinase 1; In addition to its role as a glycolytic enzyme, it seems that PGK-1 acts as a polymerase alpha cof...	0.999

**Figure 6.** This figure shows the STRING analysis of the identified proteins. String analysis indicated a significant co-expression between Triosephosphate isomerase (TPI), Protein deglycase (DJ-1), and Peroxiredoxin-2 (PRDX2). Based on STRING analysis, both FGB and RUVBL1 proteins have an independent expression pattern compared to the other identified proteins.

**Table 3.** Functional analysis of the identified proteins

Protein	Molecular function	Pathways	Proteins interaction	Biological Process
PRDX2	Peroxidase activity Protein binding Antioxidant activity	Detoxification of ROS Glucose metabolism Neurodegenerative diseases		Activation of MAPK activity response to oxidative stress removal of superoxide radicals
TPI	Isomerase activity Protein binding Methylglyoxal synthase activity	Carbon metabolism Glucose metabolism		Gluconeogenesis Glycolytic process, Methylglyoxal biosynthetic
DJ-1 (PARK7)	Redox-sensitive chaperon Transcription coactivator Redox-sensitive protease DNA and RNA binding	PI3K / Akt signaling Parkinson disease Alpha-synuclein signaling		DNA repair Synaptic transmission
RUVBL1	ATPase activities DNA helicase activities Nucleotide binding Transcription coactivator	C-MYC pathway Wnt signaling pathway Chromatin regulation / Acetylation		DNA repair DNA recombination chromatin remodeling
FGB	Protein binding ECM structural constituent	Blood clotting cascade cell adhesion		Immune response Platelet degranulation

The protein interactions are according to GeneCards database. MW: Molecular weight; pI: Isoelectric point; COV: Coverage; PRDX2: Peroxiredoxin-2; RUVBL1: RuvB-like 1; DJ-1 (PARK7): Protein deglycase; TPI: Triosephosphate isomerase; ROS: Reactive oxygen species; MAPK: The mitogen-activated protein kinase

as glucose metabolism, DNA repair, redox hemostasis, cell adhesion, and blood clotting cascade. In this regard, STRING analysis exhibited a significant co-expression between TPI, DJ-1 (PARK7), and PRDX2.

An increased level of glycolysis is believed to be a remarkable property of cancer cells due to the extended energy requirement for cell proliferation. This feature is relatively assisted by upregulation of the glycolytic enzymes, such as Triosephosphate isomerase (TPI).<sup>24</sup> TPI catalyzes the reaction to convert dihydroxyacetone phosphate into glyceraldehyde 3-phosphate, and vice versa in glycolysis and gluconeogenesis. Our results revealed a high immunoreactivity between TPI and their cognate antibodies at advanced stages of the disease. Additionally, antibody production against TPI was previously reported in the early stages of BC.<sup>25</sup> The co-expression between TPI and early-stage immunogenic proteins (DJ-1 (PARK7) and PRDX2) in our study may clarify these proteins' tendency to show a coordination in expression and probably simultaneously participate in a biochemical pathway required for cancer progression. These findings may suggest that antibodies against TPI were continuously produced from the early stages of the disease,

which can be detected even at higher stages.

Peroxiredoxins (PRDXs) are non-seleno peroxidases that catalyze the peroxide reduction of H<sub>2</sub>O<sub>2</sub> and peroxynitrite. An elevated level of ROS in the early stages of BC leads to upregulation of PRDX2. Desmetz et al. reported that autoantibodies against PRDX2 could be used for early diagnosis of invasive BC.<sup>12</sup> In accordance with these results, we also observed that PRDX2 autoantibodies are generated in early stages of BC.

DJ-1(PARK7) protein, as an oxidative stress sensor, has exhibited a functional similarity to PRDX2. DJ-1 mRNA is overexpressed in different types of cancer, such as BC, and helps cancer cells to escape from PTEN enforced cell death.<sup>26, 27</sup> Furthermore, two previous studies illustrated a high level of DJ-1 protein in sera of BC patients.<sup>28, 29</sup> DJ-1 protein undergoes various post-translational modifications (PTM), among which O-linked N-acetylglucosamine is one of the effective PTMs, enhancing oncogenic activation of DJ-1 in primary stages of BC.<sup>30</sup> LC MS/MS analysis in this study revealed different PTMs in DJ-1 sequences, such as oxidation and carbamidomethylation. Overexpression of DJ-1 in BC tumor tissues and various post-translational modifications might lead to autoantibody

production against DJ-1(PARK7) in primary stages of BC.

Based on STRING analysis, both FGB and RUVBL1 proteins have an independent expression pattern compared with the other identified proteins. Fibrinogen is a plentiful protein synthesized in the liver, whose concentration ranges from 1.5-4 g/L in human blood plasma.<sup>31</sup> Extrahepatic tissues, such as cancer cells, can produce FGB, which leads to pathological conditions. The association between coagulation factors and cancer was previously proven.<sup>32</sup> It has been also reported that high plasma fibrinogen levels are associated with cancer development and progression.<sup>33, 34</sup> BC is characterized with fibrinogen deposition without further conversion to fibrin in the tumor stroma. This new finding about tumor cells' ability suggests that extrahepatic cell-derived FGB and plasma FGB may have specific functions in the process of various diseases, including BC. In 2019, Setareh Fayazfar et al. showed that FGB could be considered as a potential plasma biomarker for early diagnosis of colon cancer.<sup>35</sup> Consistently, we detected a high immunoreactivity between FGB and their cognate antibodies in the early stages of BC.

RUVBL1 acts as a DNA-dependent ATPase, belonging to the ATPases protein family.<sup>36</sup> It is mostly overexpressed in various cancers and plays a pivotal role in oncogenic processes.<sup>37-39</sup> An increase in the expression of RUVBL1 was reported in high metastatic BC cells, and silencing RUVBL1 consequently suppressed cancer cell development in both in vitro and in vivo models.<sup>40</sup> In this study, we realized that autoantibodies against RUVBL1 were produced in the late stages of BC probably due to the aberrant expression of RUVBL1. Research has exhibited that overexpression of RUVBL1 as an immunogenic protein might be directly related to tumor progression and development. Thus, RUVBL1 could be considered as a new candidate marker for BC targeted therapy, specifically in those with advanced stages.

Multiple BC-specific AAbs have been identified for early diagnosis of the disease. Although individual AAbs have shown poor

performance for population-based screening, autoantibody panels have shown encouraging results. Modern screening digital mammography has a sensitivity of 86.9% for BC screening. None of the AAb panels reported so far for BC has qualified as standalone screening assays, but could be useful in combination with routine mammography screening. Moving these promising AAb candidates into clinical use necessitates a rigorous systematic approach. Proper study design, statistical models, extensive analytical and clinical validation, along with well-defined quantitative parameters are the necessary attributes to develop a useful AAb-based diagnostic screening tool for BC.

To date, researchers in the field of proteomics have mostly focused on comparing the proteomics pattern of BC patients with normal samples. Our study was nonetheless conducted using the SERPA method to identify immunogenic antigens in the sera sample of BC patients. Our work also detected these antigens and their autoantibodies, which can be used as a novel strategy for cancer therapy and early detection. Taken together, SERPA allows the simultaneous analysis of hundreds of proteins that are most intensely expressed in the tumor.

The technique of 2-DE; however, only detects the denatured proteins (linear epitopes) and the structural epitopes will be missed by the 2-DE. Moreover, this is a preliminary study, and the identified proteins as well as cognate antibodies should be verified with larger scale of BC patients by other methods, for instance, immunohistochemistry and ELISA, in order to explore their exact roles in early detection and clinical management of breast cancer.

## Conclusion

In summary, our results revealed that immune-proteome pattern of BC patients changes along with BC progression from primary to advanced stages, which is related to the fact that severity of immune response varies from stage to stage in BC patients. Given the identification of three immunogenic proteins (FGB, PRDX2, and DJ-1) in the early stages of BC, these proteins could

stimulate the humoral immune system to produce antibodies in the primary phase of BC; thus, autoantibodies against them may be used as complementary factors for early detection of the disease. This study also demonstrated that RUVBL1 as a late-stage immunogenic protein may play a significant role in tumor development. Therefore, targeted therapy against this protein might be considered as a new promising approach to inhibiting tumor growth.

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## Conflict of Interest

None declared.

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