ENO-1 MAY BE AN IMMUNE BIOMARKER OF $ER^+/PR^+/HER2^-$ BREAST CANCER PHENOTYPE

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Abstract - Efficient and affordable prognosis or diagnosis of the heterogeneous breast neoplasms can be achieved by the introduction of common biomarkers in all the subtypes of breast cancer (BC). In this regard, immune biomarkers have been in the spotlight of a variety researches over the years. Recently, it has been shown that ENO-1 can induce the humoral immune response in $ER^+/PR^-/HER2^-$ and $ER^-/PR^-/HER2^+$ BC patients;however, its antigenicity has not yet been studied in the $ER^+/PR^+/HER2^-$ phenotype. Hence, to check any probable similarities in the humoral immune response against ENO-1 in these 3 subtypes of breast malignancies, herein, sera of the newly diagnosed $ER^+/PR^+/HER2^-$ BC patients were used in a 2D immunoblotting approach. The immunoreactivity of ENO-1 was assessed by a dedicated image analysis platform which further approved by MALDI mass spectrometry. Results of the present study can be an initial step to introduce ENO-1 as a potential clinical biomarker candidate. 2D immunoblotting is a suitable indicator of the immunogenicity assay; hence, the antigenic characteristics reapproves by the pI and molecular weight of the protein spots and covers the bias induced by non-specific interaction in commonly-used high-throughput approaches like ELISA. Nevertheless, we strongly suggest that the results of this study must be further confirmed by more high-throughput approaches in larger populations.

Keywords - ENO-1; immunoantigen; proteome analysis; 2D immunoblotting; antigenicity; breast cancer.

I. INTRODUCTION

Breast cancer (BC) is the main cause of death in women worldwide (626,679 deaths per year) and according to the GLOBOCAN latest report (2018) each year about 2.1 million new cases are diagnosed (1). These statistics indicate that despite the enhanced functionality of the theranostic approaches and the 5-year survival of BC patients, the disease is still a serious global health concern, especially in developing countries.BC is a completely heterogeneous disease. The histological and biochemical properties of the breast neoplasms not only varies widely in different patients but also itis observable within a single case(2). Hence, more effort is needed to decipher the molecular profileof this malignancy and identification of blood-based biomarkers can be an important attempt to answer this demand. Blood-based biomarkerscan represent easily accessible criteria of the disease futures and also are valuable candidates for immunotherapy and immunodiagnostics. Many of these biomarkers have passed clinical trials and are used widely in clinics for different purposes. For instance, carcinoembryonic antigen (CEA) and mucin 1 (MUC-1) can be mentioned as the blood-based biomarkers suitable for predicting the response to therapy in BC patients and the chance of the disease recurrence. However, none of the present biomarkers are of enough sensitivity and specificity and they could not be independently used for early detection of BC(3).Thus, seeking for more effective biomarkers to introduce new ones is still promising. Researchers worldwide believe that increasing the biomarker knowledge canimprove clinical potency for sure. Antibodies against tumor-associated antigens (TAAs) appear in serumeven far before cancer diagnosis.They remain in sera for several years; so, theycan be highly potent and promising biomarkers (4). Antitumor autoantibody-based assessments are simple and easy to use, and could also provide great opportunities for early detection of BC(5). Moreover, tumor antigen specific antibodies could function as antitumor agents because they could either activate other immunological mechanisms or disrupt mechanisms crucial for cell proliferation of tumor cells (6). Prohibitin, Alpha 2HS-glycoprotein (AHSG) and hnRNPK can be mentioned as some examples of BC potent antigens (7-9) each with its specific sensitivity and selectivity. The latest generation of the diagnostic antigenic kits is based on the design of combined antigenic panels(consist more than a single antigen) to enhance the efficacy of detection (10).

Recently, ENO-1 or Enolase-α, a TAA capable ofeliciting humoral immune response.has drawn attentions in a number of researches. Several studieshave also revealed the immunogenicity of ENO-1 in different types of cancerssuch as prostate, gingivobuccal complex (GBC) and melanoma (11-13).As a glycolytic enzyme,ENO-1, may act as an accelerator for the proliferation of cancer cells in hypoxic situationsvia aerobic glycolysis or Warburg effect (14).Inhibition of ENO-1 expression can also be of the therapeutic value.It has been shown that suppressing the ENO-1 expression can lead to the enhanced sensitivity of ER^+BC patients to tamoxifen. Thissuggests that this metabolic enzyme plays a pivotal role in the progression of cancer (15). ENO-1 can induce a strong immunogenicity in $ER^+/PR^-/HER2^-$patients compared with $ER^-/PR^-/HER2^+$cases; but its immunogenicity in $ER^+/PR^+/HER2^-$has not been reported. Therefore, herein, we evaluated the

antigenicity of ENO-1 in this subtype of BC using a proteomic-based approachto demonstrateif this protein could induce the humoral immune response in BC patients of $ER^+/PR^+/HER2^-$ phenotype.

II. MATERIALS AND METHODS

Serum samples collection and preparation

Blood samples were obtained from $ER^+/PR^+/HER2^-$, BC patients at the time of the diagnosis. 3volunteer patients (mean age: 46± 3.6 years) and 3 healthy controls (mean age: 52.66±3.05 years) provided written informed consent and enrolled in the experiment. To obtain sera suitable for sensitive immunoblotting approach, blood samples were then centrifuged at 5000 rpm for 7 min to exclude blood cells. The sera were then stored at -80°C until use.

Cell culture and proteome extraction

The $ER^+/PR^+/HER2^-$ cell line (T-47D) were purchased from National Iranian Biological Resource Center. Cells were then cultivated at 37°C under 5% CO_2 in F12 high glucose DMEM media (Sigma-Aldrich, Germany) supplemented with 10% (V/V) fetal bovine serum (Sigma-Aldrich, Germany).

In order to extract total proteome, the T-47D cells were washed with cold phosphate-buffered saline (PBS) (Sigma-Aldrich, Germany), and dissolved in lysis buffer (7 M urea, 2 M thiourea, 50 mM DTT, 4% CHAPS, 2% Ampholytes (Bio-Rad, Hercules, CA), supplemented with 5 mM protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland)and DNase/RNase solutions (Fermentas, Burlington, Canada)). To increase the proteome yield, The lysate was purified by acetone-methanol method mentioned in our previous work(16). The lysate was then centrifuged, and proteome concentration determined by Bradford assay.

Two dimensional immunoblotting

Before the assessment of immunoreactivity, T-47D proteome content was separated 2 dimensionally; first by their pIs and second by their MWs. Isoelectric focusing (IEF) was run in the PROTEAN® II xi Cell System (Bio-Rad). In brief, for each triplicate experiment, a suitable amount of T-47D proteome was loaded onto an Immobiline DryStrip gel (IPG) (BioRad Laboratories, Hercules, CA, USA, 17 cm long, pH 3-11 NL). The proteins were then focused at their pI in a gradually increased electric potential up to the 10 kV and 80 kVh. IPG strips were then subjected to the SDS polyacrylamide gel electrophoresis to perform second separation step according to the proteins MWs in the well-establishedO'Farrell system (14, 17). The overall 12 2D patterns (of case and controls) were then divided into two identical groups; one of which was stained by silver nitrate and the other was electrically transferred onto the PVDF membranes (Immobilon-PSQ, Merck, Darmstadt, German) for immunoblotting. For this purpose, PVDF membranes were blocked in a solution of 2% BSA in TBS for 2 h. The membranes were then incubated in the sera samples (1:1000 in TBS containing 1% BSA) overnight at 4°C. To make immunoreactive protein spots visible, a colorimetric blotting assay using alkaline phosphatase-conjugated secondary goat anti-human antibody was performed. To this end this secondary antibody was diluted to the ratio of 1:10000 in TBS containing 1% BSA and applied to the sera treated membranes for 1h at RT.

Evaluation of the ENO-1 immunoreactivity in a $ER^+/PR^+/HER2^-$ BC phenotype

The high quality 3D images of the gels and blots were obtained by GS-800 densitometer (Bio-Rad). Image Master™ 2D Platinum software, Melanie 6 (GE healthcare) was the applied for precise spot detection and alignment between two groups of patients and healthy controls. In order to determine the possible immunoreactivity of ENO-1 in candidates of $ER^+/PR^+/HER2^-$ BC phenotype. The pI and MW of ENO-1 was determined using ExPASy pI/Mw tool (https://web.expasy.org/compute_pi/). These values were then entered into the Melanie pI/Mw calculator to seek any possible immunereactivity of ENO-1 and make a comparison with the controls response.

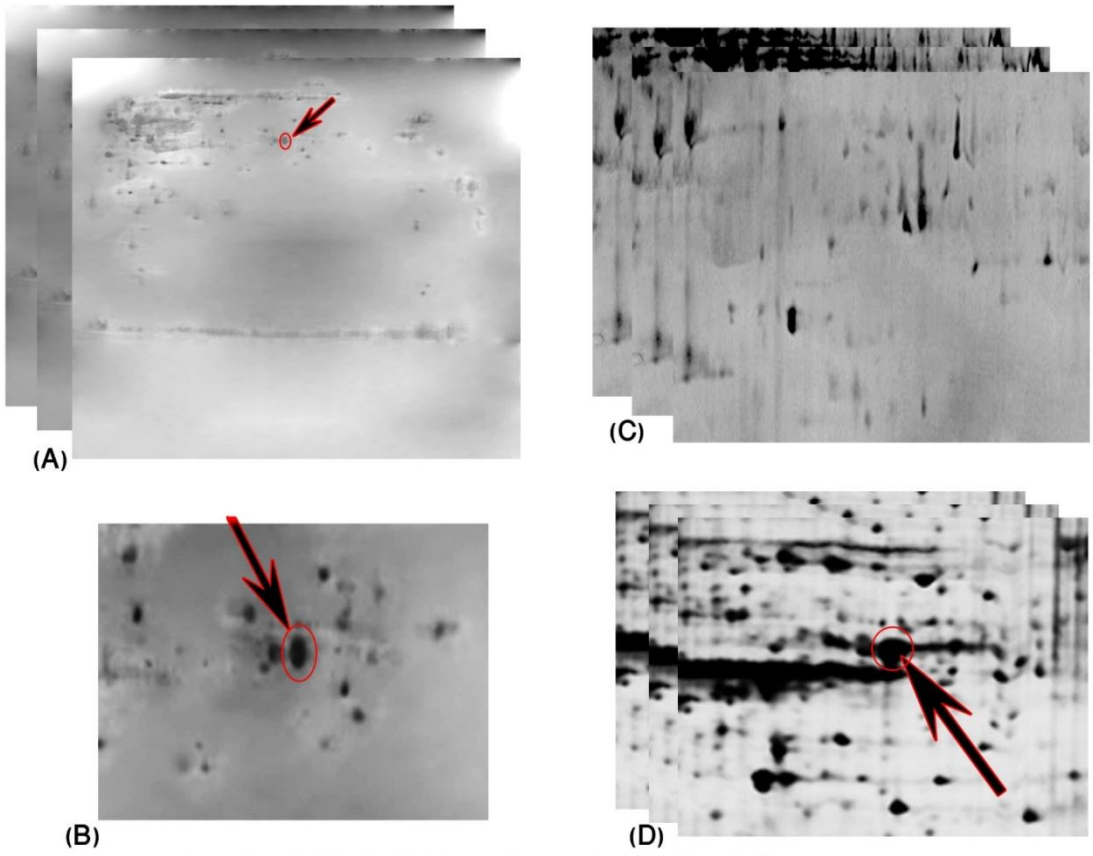
Mass spectrometry verification of ENO-1 identity

To insure the identity of ENO-1 in its corresponding position in 2D proteome patterns, the probable ENO-1 spots were excised manually from the gels and subjected to the matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry analysis. This experiment was performed according to the Proteomics & Analytical Biochemistry Labs, University of York, UK, protocol.

III. RESULTS AND DISCUSSION

Western blotting techniques provide suitable platforms to early assessment of the immunoreactivity of proteins compared to the more high-throughput approaches like enzyme-linked immunosorbent assay (ELISA); since, these techniques provide more additional conformation steps to distinguish non-specific interactions. Indeed, the antigenic characteristics can be further verified by the molecular weight of the antigen. This is more confident in the case of 2D immunoblotting in which both pI and MW of a spots can be assigned. Hence, the approach is more suited for the onset of a research program. Therefore, in the present study, immunoreactivity of ENO-1 in $ER^+/PR^+/HER2^-$ BC phenotype was assessed by a 2D immunoblotting approach. To our knowledge it is first report of the ENO-1 immunogenicity in $ER^+/PR^+/HER2^-$ BC phenotype. Results of our 2D immunoblotting approach and the position of the probable

immunoreactive ENO-1 is presented in fig. 1. and as it is evident in the figure ENO-1 has elicit a strong humoral immune response in all the 3 replicates. Fortunately results of the mass spectrometry verification step further confirmed that the immunogenic spot depicted in fig. 1 is ENO-1.



(E)

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1	1143.6401	1142.6329	1142.6084	21.4	0	64	4.4e-05	1	U	R.IGAEVYHNLK.N
6	1805.0205	1804.0132	1803.9366	42.5	0	33	0.028	1	U	R.AAVPSGASTGIYEALELR.D

Figure 1. Results of 2D proteome profiling, 2D immunoblotting and Mass spectrometry. A and C:2D immunoblot patterns of T-47D profiles treated with the sera obtained from $ER^+/PR^+/HER2^-$ BC patients and healthy controls, respectively. B: a zoomed view of the ENO-1 reactive antigen adopted from the A panel. E: Result of the peptide mass analysis of ENO-1 obtained from MALDI-TOF expriment.

CONCLUSION

BC is an extremely heterogeneous neoplasm. Hence, seeking a common biomarker which shows the same behavior in all the subtypes of the disease can provide a promising platform to design more accurate and sensitive diagnostic panels. Thus, considering the overexpression and immunogenicity of ENO-1 in gene, mRNA and protein levels in some types BCs as well as a number of other tumor types (18-20); herein, for the first time we planned to seek ENO-1 behavior in the $ER^+/PR^+/HER2^+$ BC phenotype. We showed that ENO-1 can also be an immunogenic biomarker for $ER^+/PR^+/HER2^+$ patients. A result which must be further approved by more high-throughput approaches in larger populations. We also suggest the use of the methodology applied in the present research to obtain a more comprehensive map of all the antigens involved in eliciting the humoral immune response to the breast neoplasms.

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