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RESEARCH ARTICLE

SELDI-TOF-MS Profiling of Metastatic Phenotype in Histopathological Subtypes of Breast Cancer

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Abstract: ***Background***: Early detection of breast cancer is a key to the success of breast cancer management. Serum proteome analysis using Surface-Enhanced Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (SELDI-TOF-MS) generates useful information that can be utilized to describe exclusive prognostic and diagnostic biomarkers.

Objective: This study aimed to use proteomics and bioinformatics to identify new biomarkers during the metastatic process of breast cancers that were classified as invasive lobular cancer or invasive ductal cancer.

Method: Blood samples from 64 breast cancer patients [36 with invasive ductal cancer (14 of whom were lymph node positive); 28 with invasive lobular cancer (8 of whom were lymph node positive] were analyzed using IMAC 30 protein chips. The data acquired from the spectra were processed with univariate statistical analysis (Protein Chip Data Manager Software).

Results: One-hundred-eighteen clusters were generated from the individual serum samples. Thirty-six proteins of the metastatic phenotype were found to be diagnostically accurate in cluster analysis. In the breast cancer group, a single candidate peak (m/z 1090.8) that was able to discriminate the metastatic progression was identified as a metastatic phenotype marker. Fifteen protein peaks were identified as markers to separate the histopathological subtypes as either invasive ductal cancer or invasive lobular cancer.

Conclusion: In recent years, proteomic methods have rapidly become widespread in breast cancer research. This study revealed the pattern of a group of proteins that were not previously identified and are recommended as candidate markers to diagnose metastatic progression.

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1. INTRODUCTION

The most widespread form of cancer in developed and developing countries is breast cancer. An early diagnosis is one of the important aspects in the optimal treatment of breast cancer. The use of proteomic tools helps to identify exclusive prognostic and diagnostic biomarkers and enables the detection of quantitative and qualitative patterns of proteins in cerebrospinal fluid, saliva, urine, blood and other bodily fluids and tissues. Serum proteome analysis generates useful information that can be utilized to identify potential biomarkers associated with cancer progression.

The prognosis of breast cancer is defined according to the quantity of the lymph nodes, size of the tumor, hormone receptor status and histological grade. The histological grade and histological type consider the growth patterns and degree of differentiation of the tumor and are used to morphologically

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classify carcinomas [1]. Clinical and pathological classification is important in terms of classifying breast cancer patients for the purposes of assessing their prognosis and determining an appropriate treatment [2]. Invasive lobular carcinoma in the breast, which constitutes up to 15% of all breast cancer patients, is a common morphological subtype of breast cancer, while invasive ductal carcinoma (IDC), in its most aggressive form, represents 70-80% of all breast cancer patients and has a poor prognosis [3, 4]. Molecular techniques are increasingly being used to clarify the classification of breast cancer. These techniques include CT scanning, X-ray, breast sonography and mammography. Nevertheless, these tools either do not have the accuracy to specify microangiogenesis in the early stages of breast cancer or they cannot adequately resolve the spatial detail to distinguish very small breast cancers [5-7]. For example, when using a mammogram, it may be harder to detect ILC than IDC.

Plasma and serum are ideal samples for the detection of biomarkers in clinical studies due to the easy access of blood samples using routine drawing techniques [8, 9]. In recent years, proteomic research methods have been widely used to more accurately define breast cancer patients. The use of quantitative biomarker-based tests yields more objective results than clinical assessment because there is much less observer variation and laboratory quality assurance protocols provide a higher level of reliability and reproducibility. When a lesion cannot be detected using mammography, plasma and serum markers can still point to a breast tumor in its early stages. Transcriptomic analysis has been used to identify many genes that are either up- or down-regulated after treatment when investigating and predicting the response of a tumor to treatment. Peptidome analysis based on the SELDI-TOF and MALDI-TOF-MS screening methods is an encouraging approach with high-throughput capabilities that could help to identify new potential biomarkers in serum or other body fluids [10]. When analyzing a complex biological specimen, it is possible to achieve accurate and high-throughput protein profiling with SELDI-TOF-MS. For this reason, SELDI-TOF-MS may be able to increase the potential detecting early stage breast cancer by enabling the discovery of new and better biomarkers [9, 11].

This study aimed to provide a proteomics and bioinformatics approach to define metastatic progression of histopathologically classified IDC and ILC in the early stages of breast cancer at the molecular level and to determine the clinical practicality of new biomarkers.

2. METHODS

2.1. Patients

This prospective study was performed in the Ataturk Training and Research Hospital First Surgical Department. The study included 64 patients with breast cancer. Among these patients, 36 had IDC (14 lymph node-positive) and 28 had ILC (8 lymph node-positive). Patients undergoing surgery had blood samples taken immediately prior to surgery; all other patients had blood samples taken before treatment. Mammograms and breast ultrasounds for all patients were performed before treatment. Diagnoses were confirmed by pathology tests. Additional clinical information for patients with cancer included the cell type, tumor size, lymph node, and estrogen and progesterone receptor status. The study was performed in accordance with the principles expressed in the Declaration of Helsinki and was approved by a local ethics committee.

2.2. Serum Samples

Blood samples were collected in a sitting position during surgery and treatment and after 12 hours of fasting *via* routine venipuncture into eight-milliliter gel-containing vacuum tubes (BD™ P100 Blood Collection System for Plasma Protein Preservation, Franklin Lakes, NY, USA). Serum samples were aliquoted into 250 µL samples and stored at -80ºC until analysis.

2.3. Pathology

The most descriptive block was picked in each malignant subject. P53 (Dako Clone Do7; Denmark), progesterone receptor (PR) (Neomarkers RM 9102-S, USA), c-erbB-2 (Labvision Clone SP 3 7 ml USA), estrogen receptor (ER) (Novocastra RT4-ER-6F11 7 ml, UK), and the Ki-67 proliferation marker (Dako Clone MIB1, Denmark) were purified via the streptavidin–biotin method. After diluting at 1/100, 1/25 and 1/75, respectively, PR, P53 and the materials used for Ki-67 proliferation were applied. Mayer's hematoxyline was utilized as an opposite stain, and diaminobenzidine was utilized as a chromogen material. To evaluate PR, ER and P53, the nuclear staining intensities were classified as "weak" (1+), "moderate" (2+) or "intense" (3+), and their percentages were calculated. Based on membranous staining in invasive tumors, c-erbB-2 was scored at 4 levels. Ki-67 was calculated based on the number of areas where nuclear staining was the most intense.

2.4. Serum Protein Profiling

IMAC 30 protein chips loaded with Cu^{2+} were employed to identify the proteins in serum. The chips were activated by subjecting each sample to 50 µL of 100 mM $CuSO_4$ for five minutes. The samples were then cleaned with distilled water. Next, they were washed three times for ten minutes each using 200 µL of binding solution (500 mM NaCl, 100 mM NaH_2PO_4/NaOH, pH 7.0). Each serum sample was diluted with dilution buffer [9 M urea, 50mM Tris/HCl (pH 9), CHAPS (0,2% (w/v)] at a ratio of 5:1 and vortexed on ice. Ten microliters of this mixture was then added to 100 µL of binding buffer. Each spot was mixed with 100 µL of the serum mixture and then incubated on a horizontal shaker (900 rpm) for 60 minutes. The chips were washed four times with 200 µL of binding solution (each on a horizontal shaker, for ten minutes). The wells were then quickly rinsed with 1 mM HEPES (pH 7.0) and dried at room temperature. After adding 1 µL of matrix solution (sinapinic acid solution, 50% saturated, in 50% acetonitrile, 0.5% trifluoroacetic acid) to each well, the wells were dried at room temperature. This procedure was repeated two times. The chips were automatically loaded into the PBS IIc SELDI-TOF (Ciphergen, Biosystems Inc., Fremont, CA, USA) device for SELDI-TOF-MS analysis. To measure the reproducibility and stability of the mass spectra, the IMAC-Cu purification process for all

samples was repeated three times. For each SELDI protein chip assay, variations within an assay were determined by using SELDI profiling of ten aliquots from the same serum sample. After placing the chips in the Protein Biological system II-C mass spectrometer reader (Ciphergen Biosystems, Inc.), TOF spectra were generated by averaging 192 laser shots at an intensity of 220 with a detector sensitivity of nine. The range collected was between 1,000 and 50,000 Da, and a maximum of 200,000 Da was obtained. The instrument was externally calibrated using an all-in-one peptide molecular mass standard (Ciphergen Biosystems, Inc.) including six peptides: somatostatin (1637.903 Da), human ACTH (1-24) (2933.500 Da), (Arg8)-vasopressin (1084.247 Da), hirudin BKHV (7033.614 Da), dynorphin A (2147.500 Da) and bovine insulin B-chain (3495.941 Da) (Supplementary Fig. **1**). We were able to obtain a mass accuracy of 0.08% using this system.

2.5. Bioinformatics

The Protein Chip Data Manager Software (Ciphergen Biosystems) was used for spectral data collection, data processing and univariate statistical analysis. The intensities of all spectra were internally calibrated and were normalized to the total ion flow. Automatic baseline correction was used to reduce the effects of background. The default value for data filtering was set as 0.2 times the expected peak width. Mass peaks considered to be eligible had a signal/noise >5 and were within a 0.3% of the cluster mass window. For each peak cluster, the one way Mann-Whitney U test was used to perform inter-group comparisons, and p values were calculated. The statistical significance value was set at $p<0.05$. For each peak cluster, the area under the receiver-operator characteristic curve (ROC) (AUC) were calculated. The peaks that presented statistically significant differences in the one-way statistical analysis were reassessed and confirmed until no incorrect peaks remained. The training set was formed from 118 clusters. In our study, the cluster mass was set to 0.5% and the signal-to-noise ratio for the second pass was set to one.

2.6. Statistical Analysis

Raw data were transferred to the statistical software package ProteinChip Data Manager to perform univariate statistical analysis. All spectra were internally mass-calibrated, and the peak intensities were normalized based on the total ion flow. Peak aggregation and selection were conducted by excluding the very low mass region (0-1,500 Da), which overlapped with peaks from single-photon absorptiometry. The Mann-Whitney U test was used to compare each peak cluster. Statistical significance was defined as $p<0.05$. All of the peaks exhibiting statistically significant differences in one-way statistical analysis were reassessed and confirmed until no incorrect peaks remained. The ROC-AUC values in down-regulated proteins were expressed as (1-value). The statistical software SPSS 21.0 (SPSS, Inc., Chicago, IL, USA) was used to perform all statistical analyses.

3. RESULTS

3.1. Demographic and Clinicopathological Characteristics of Breast Cancer Patients

The demographic and clinicopathological characteristics of the patients are presented in Table **1**. Sixty-four breast cancer patients were included in the study. There were 36 patients with IDC (14 lymph node positive) and 28 patients with ILC (8 lymph node positive). The mean ages of the IDC patients (n=36) and ILC patients (n=28) were 54.33±11.64 and 53.33±6.26, respectively. Serum samples from patients were evaluated by SELDI-TOF to determine whether lymph node-positive and lymph node-negative patients could be distinguished by their proteomic patterns.

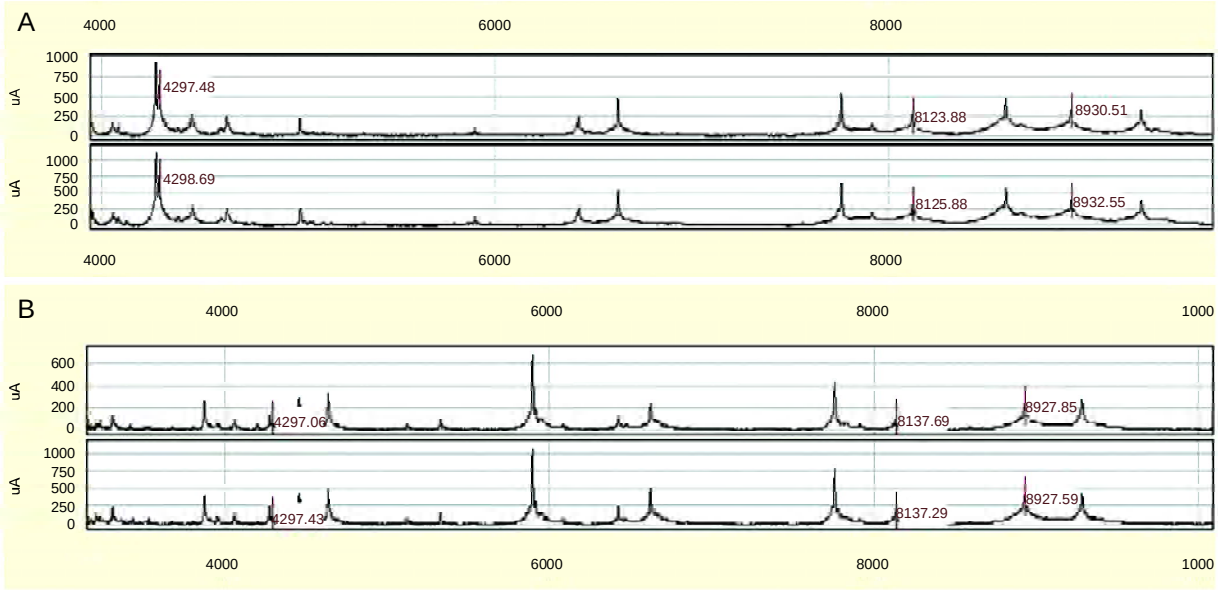


Fig. (1). SELDI-TOF-MS peak images, (**A**) breast cancer and (**B**) healthy control. Serum proteomes were visualized between 4000-10000 Da.

Table 1. Demographic and clinicopathological characteristics of breast cancer patients.

Characteristics	Invasive Ductal Carcinoma	Invasive Lobular Carcinoma
N	36	28
Age (Mean ± SD)	54.33 ± 11.64	53.33 ± 6.26
CA15-3 (U/mL)	19.17 ± 5.0	32.70 ± 30.77
ER (+)	62.5%	66.6%
PR (+)	56.25%	50%
c-erbB-2 (+)	6.7%	0%
Ki-67 (+)	57.14%	0%
P53 (+)	6.94%	0%

Abbreviations: CA15-3, Carbohydrate antigen 15-3; ER, estrogen receptor; PR, progesterone receptor; c-erbB-2, HER-2/neu; p53, tumor suppressor 53.

3.2. Peak Detection Based on IMAC-Cu

Purification of serum samples taken from cases was performed using MB-IMAC-Cu (Fig. **1**). Spectra analysis using ClinProTools software 2.2 identified proteomic patterns that rendered a clear distinction between patients with IDC and patients with ILC. The protein intensity differences between IDC and ILC patients were determined using the Mann-Whitney U test. The test was adjusted for contradicting variables in a multivariate logistic regression model.

3.3. Serum Protein Profiling of IDC and ILC Patients

The training set identified 180 peaks per spectrum that were found to have masses ranging from 2-20 kDa. Mass spectrometry analysis indicated that there were 36 peaks with signal-to-noise ratios >5 that were detected between mass to charge (m/z) 2,000-20,000 in serum samples from the two histopathological groups. All peaks were incorporated into a k-nearest neighbor genetic algorithm. The model consisted of a set of 15 potential biomarker peaks. These fifteen peaks were statistically significant ($p<0.05$) for their ability to identify histopathological types of breast cancer. Eight up-regulated and seven down-regulated proteins were identified among these peaks. The specificity and sensitivity of the diagnostic validity of the 15 peaks were 100% and 94%, respectively (Table **2**).

By using SELDI-TOF-MS, the comparison of patients with IDC and ILC breast cancer resulted in eight up- and seven down-regulated protein peaks. Among 118 protein peaks, 15 protein peaks were determined to be significant in binary logistic regression, and these peaks were classified as IDC and ILC. The receiver-operating characteristic (ROC) for these peaks were area-under-the-curve values of between 0.773-0.710 for up-regulated and 0.872-0.802 for down-regulated proteins. All of the down-regulated peaks were at a significance level of $p<0.001$; the significance of the other peaks varied from $p<0.001$ to $p<0.05$.

Table 2. The diagnostic accuracy and the mean level of differentially expressed proteins between invasive ductal carcinoma and invasive lobular carcinoma patients.

m/z	Trends	p	AUC*	IDC (n: 36)	ILC (n: 28)
				Intensity (Mean ± SD)	Intensity (Mean ± SD)
3883.8	up	0.001	0.773	288.695 ± 44.352	176.431 ± 60.761
2951.8	up	0.001	0.773	152.736 ± 147.902	28.639 ± 36.434
5944.7	up	0.001	0.758	86.634 ± 83.645	15.965 ± 18.768
1090.8	up	0.003	0.726	109.915 ± 113.637	39.059 ± 42.650
5887.9	up	0.010	0.726	116.741 ± 11.667	31.16 ± 8.822
7785.8 (BC2)	up	0.002	0.726	168.772 ± 97.741	98.604 ± 39.682
2211.6	up	0.005	0.716	191.117 ± 47.386	123.483 ± 28.564
1563.6	up	0.014	0.710	96.183 ± 56.892	64.196 ± 40.979
3145.3	down	< 0.001	0.872	44.21 ± 32.386	112.248 ± 52.494
2634.3	down	< 0.001	0.826	27.819 ± 20.563	81.630 ± 68.697
4300 (BC1)	down	< 0.001	0.825	246.965 ± 161.543	502.219 ± 219.905
8601.7	down	< 0.001	0.825	96.697 ± 94.899	260.047 ± 127.123
5039.8	down	< 0.001	0.799	27.494 ± 46.435	46.381 ± 22.460
3687.1	down	< 0.001	0.809	42.799 ± 18.485	79.228 ± 37.724
2140.9	down	< 0.001	0.802	43.611 ± 44.330	117.638 ± 71.424

Abbreviations: AUC, area under curve; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; SD, standard deviation. *AUC was defined as 1 for decreasing markers.

Table 3. Protein biomarker of metastatic lymph nodes (m/z 1090.8).

m/z	Trends	p	AUC*	LN-Positive (n: 22)	LN-Negative (n: 42)
				Intensity (Mean ± SD)	Intensity (Mean ± SD)
1090.8	Down	0.031	0.727	44.44 ± 37.6	108.02 ± 29.9

Abbreviations: AUC, area under curve; LN, lymph node; SD, standard deviation. *AUC was defined as 1 for decreasing markers.

Table 4. Proteins of metastatic phenotype in invasive ductal carcinoma patients.

m/z	Trends	p	AUC*	Invasive Ductal Carcinoma	
				LN-Negative Intensity (Mean ± SD)	LN-Positive/Metastasis Intensity (Mean ± SD)
1531.9	up	0.005	0.884	72.89 ± 53.9	210.95 ± 99.5
2211.6	up	0.007	0.884	191.12 ± 17.4	665.37 ± 85.1
2081.5	up	0.021	0.829	99.90 ± 12.2	181.27 ± 18.05
2230.9	up	0.050	0.720	40.83 ± 32.4	159.94 ± 18.4

Abbreviations: AUC, area under curve; LN, lymph node; SD, standard deviation. AUC* was defined as 1 for decreasing markers.

3.4. Serum Protein Profiling for Marker of Metastatic Phenotype

Thirty-six protein peaks were identified and composed a valid diagnostic pattern. When we compared the peaks generated from lymph node (LN)-positive (n=22) and LN-negative (n=42) cancer patients, we obtained only one down-regulated peak at m/z 1090.8 with an AUC value of 0.727 ($p<0.05$) (Table **3**).

This peak was identified as a significant defining marker of the metastatic phenotype. The sensitivity of the MB-IMAC-Cu for IDC patients with and without lymph node metastasis model included a set of four potential biomarkers (m/z: 1531.9, 2081.5, 2211.6, and 2230.9); the AUC values were 0.884, 0.829, 0.884, and 0.720, respectively, and all of the biomarkers were up-regulated metastatic phenotype peaks. Their specificity and sensitivity were 100% and 76%, respectively (Table **4**).

To identify effective biomarkers to diagnose IDC patients as LN-negative or LN-positive, the analysis resulted in four up-regulated peaks. One of the peaks had a significance level of $p<0.005$ (AUC 0.884), while the rest had significance levels of $p<0.05$ (AUC 0.884-0.720).

Ten phenotypic indicator peaks in ILC patients with and without lymph node metastasis are presented in Table **5**. Analyses of the ILC patients resulted in ten peaks; one with a significance of $p<0.05$ (AUC 0.824) and nine with significances of $p<0.005$ (AUC 0.986-0.801). Among these peaks, two were down-regulated and eight were up-regulated. The specificity and sensitivity were both 95%.

4. DISCUSSION

Metastatic breast cancer is the second most frequent cause of cancer-related death in women [12, 13]. Obtaining a diagnosis before metastasis is important since a significant portion of breast cancer deaths are caused by metastasis. Therefore, any early biomarkers that can identify metastasis will help to reduce breast cancer mortality. The most highly validated tissue-based markers for breast cancer include ER, PR, HER-2, uPA, and PAI-1, which have been routinely used to diagnose breast cancer patients [14, 15]. Since accessing tissue markers typically requires biopsies, a serum- or plasma protein-based screening test is more favorable in many diseases, including breast cancer. Indeed, many studies have been carried out over the last decade for the purposes of identifying serum biomarkers [16]. However, there are not yet well-defined and suitably validated biomarkers for the early detection of breast cancer. Thus, the present study aims to identify serum biomarkers that can classify the histopathologic subtypes of breast cancer and to identify new serum protein biomarkers that can help in the early detection of breast cancer.

In a review by Malhotra *et al.*, when evaluating the future use of biomarkers that utilized molecular-based techniques, the authors could not identify a clear reason to adopt one molecular marker or parameter over another to functionally classify breast cancer [17]. However, several SELDI-TOF-MS peaks, albeit not structurally identified, have been reported to be able to distinguish whether a serum or plasma sample is from a breast cancer patient or healthy individual [18-20]. Furthermore, protein profiles of healthy women were found to be similar to those of women with breast cancer in screenings [21].

To classify IDC and ILC breast cancers, we identified 15 protein peaks with high specificity and sensitivity. One of the identified peaks was found to be specifically increased in patients with node-positive breast cancer.

For the purpose of distinguishing LN-positive and LN-negative cancer samples, the protein profiles from IDC patients yielded sensitivity and specificity values in the range

Table 5. Proteins of metastatic phenotype in invasive lobular carcinoma patients.

m/z	Trends	p	AUC*	Invasive Lobuler Carcinoma	
				LN-Negative Intensity (Mean ± SD)	LN-Positive/Metastasis Intensity (Mean ± SD)
5132.7	up	0.020	0.986	30.48 ± 7.6	44.77 ± 10.38
2211.6	up	0.005	0.978	123.48 ± 28.6	665.37 ± 85.1
6080.5	up	0.005	0.919	12.44 ± 6.8	40.47 ± 20.89
8115 (BC2)	up	0.032	0.882	58.93 ± 18.07	102.85 ± 45.8
8601.7	up	0.007	0.882	260.05 ± 127.1	48.07 ± 40.91
4300 (BC1)	up	0.032	0.824	208.90 ± 15.84	502.59 ± 29.9
11738	up	0.039	0.824	10.79 ± 7.7	3.42 ± 1.80
3066.5	up	0.039	0.801	25.56 ± 12.7	41.32 ± 10.4
8142.7	down	0.009	0.941	216.50 ± 88.4	80.01 ± 27.95
2864.3	down	0.049	0.824	111.235 ± 44.5	71.12 ± 16.7

Abbreviations: AUC, Area Under Curve; LN, Lymph Node; SD, Standard Deviation. *AUC was defined as 1 for decreasing markers.

of 76-100%. A similar analysis of ILC patients resulted in ten protein peaks with a specificity and sensitivity of 95% for distinguishing ND+ and ND- cancer samples. When comparing breast cancer subtypes, one significant peak (m/z 1090.8) was obtained from the IMAC-Cu surface that was down-regulated in LN-positive cancer but was up-regulated in IDC patients.

CONCLUSION

In conclusion, the protein peaks identified by SELDI-TOF-MS may have the potential to be used in diagnosing metastatic progression in different histopathological classes of breast cancer. Furthermore, this study also shows that protein markers that determine metastatic progression in different histopathological classes of breast cancers have different distributions. Additional studies on SELDI-TOF analysis of serum samples will help to clarify the use of biomarkers to identify metastatic progression in all histological types of breast cancer.

LIST OF ABBREVIATIONS

AUC = Area Under the Curve

CA15-3 = Carbohydrate Antigen 15-3

c-erbB-2 = HER-2/neu

ER = Estrogen Receptor

IDC = Invasive Ductal Cancer

ILC = Invasive Lobular Cancer

LN = Lymph Node

p53 = Tumor Suppressor 53

PR = Progesterone Receptor

ROC = Receiver-operating Characteristic

SD = Standard Deviation

SELDI-TOF-MS = Surface-enhanced Laser Desorption/ Ionization Time-of Flight Mass Spectrometry

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Ataturk Training and Research Hospital Ethics Committee of Katip Celebi University (Izmir, Turkey).

HUMAN AND ANIMAL RIGHTS

No animals were used in the study. The study was performed in accordance with the principles expressed in the Declaration of Helsinki and was approved by a local ethics committee.

CONSENT FOR PUBLICATION

Informed consent were taken from all participants.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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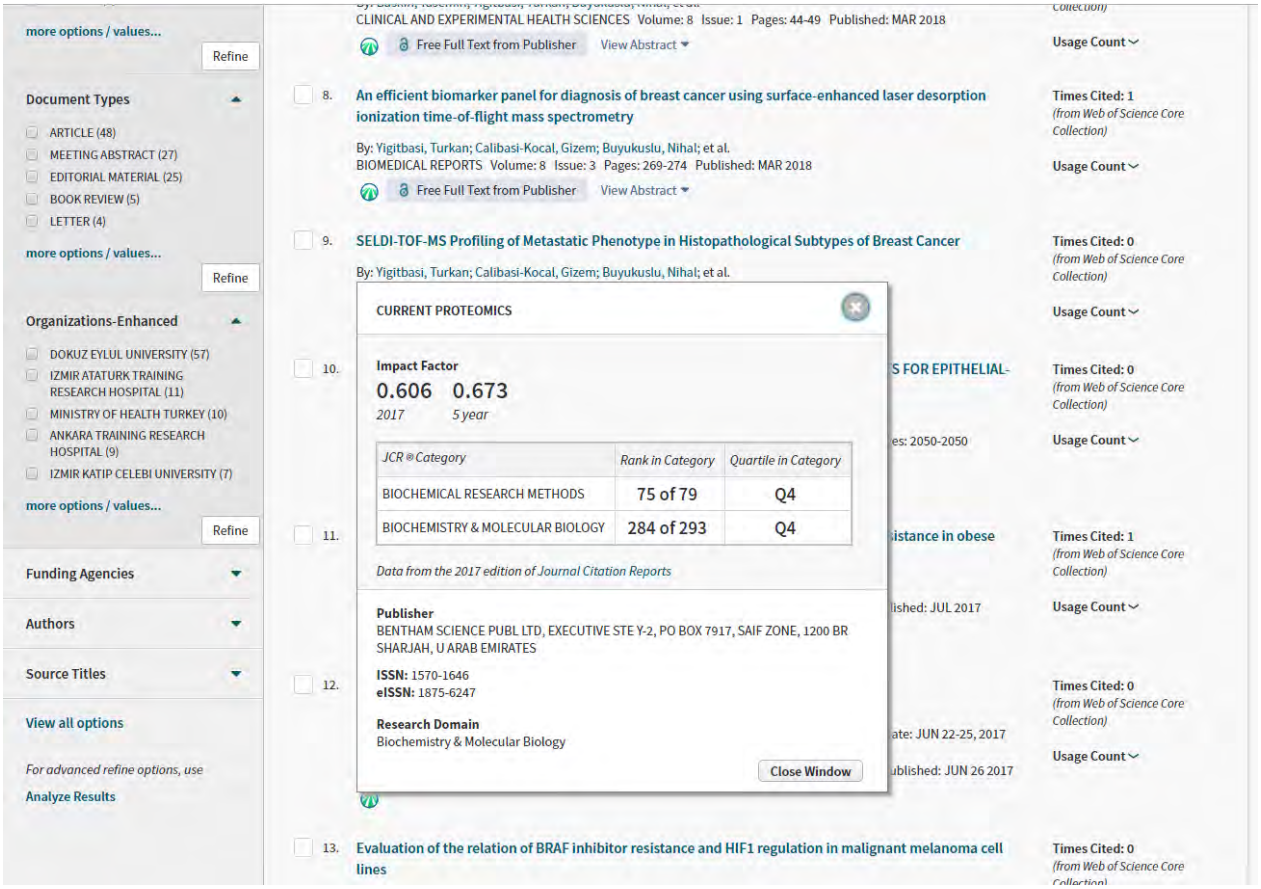
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