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ORIGINAL ARTICLE

Nectin-2 and Nectin-4 Adhesion Molecules in Patients with Breast Cancer

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OBJECTIVE
Evaluation of the nectin-2 and nectin-4 protein and mRNA expression levels is aimed in this study, with concerning diagnostic and predictive value in breast cancer patients.

METHODS
Sixty patients with pathologically and radiologically verified breast cancer who were treated at the Istanbul University, Institute of Oncology, between 2017 and 2018 are included in the study. Circulating nectin-2 and nectin-4 protein levels were evaluated by solid-phase enzyme-linked immunosorbent assay (Abbkine Scientific Co., Ltd.). For analyzing nectin-2- and nectin-4-specific mRNA in sera of the patients, circulating cell-free RNA was extracted from serum using a monophasic phenol and guanidine thiocyanate solution (Roche, Mannheim, Germany), according to the manufacturer's protocol.

RESULTS
The median age of patients was 53 years. The mean tumor size was 30.21±17.32 mm. Forty-one patients were in the luminal group. Lymph node involvement was detected in 25 patients. The nectin-4 expression level was statistically significantly higher in those with Ki-67 ≥30 and those with positive distant metastasis compared to the other group. In addition, nectin-2 expression was higher in patients with Grade 3 tumors.

CONCLUSION
High levels of nectin-2 and nectin-4 expression in the serum of patients correlate with poor disease characteristics of breast cancer.

Keywords: Adhesion molecules; breast cancer; histology; nectin; pathology.

Introduction

Breast cancer comes first as the cancer-related cause of death in the female population; despite the effective screening programs which result in early diagnosis. The survival rate is high when the disease is diagnosed in the early stage; however, 20-30% of local breast cancer cases will be progressed to the metastatic stage. [1] The progressive potential of the disease is based on disease and patient characteristics such as age, receptor status, and tumoral invasiveness. Aggressive disease courses in patients with worse pathological

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characteristics underlines the demand for enhanced methods to predict the patients who are a candidate for aggressive and novel targeted strategies for treatment. Elevated plasma CA-15-3 levels are tested frequently for surveillance of recurrent breast cancer; however, its correlation with patient characteristics and prediction of treatment modalities are not validated and routine analysis does not recommend in current guidelines. [2] At present, the most commonly used markers to guide the treatment options are hormone receptor and HER2-neu status on pathological material. However, disease courses are different even in patients with the same histologic properties.[3] There is a need for discovering new biomarkers to explain these differences.

Serum biomarkers are extensively investigated in cancer patients to close the gaps in diagnostic and predictive markers. Nectins are a kind of immunoglobulin-like homo-heterophilic cell adhesion proteins that maintain intercellular adherence and tight junctions.[4] There are four nectin proteins that described; nectin-1 and -2 are mainly found in adult somatic tissues, nectin-3 is expressed mainly in reproductive organs such as testes and placenta, and also nectin-4 expression is naturally limited to the placenta.[5-7] On the other hand, binding characteristics are different for each nectin subgroup.[6] There have been reports of expression of nectin-4 in ductal breast carcinoma and lung adenocarcinomas.[8-10] Nectin-2 is less frequently studied in oncologic patients; Oshima et al.[11] have been shown the elevated expression in breast cancer tissues. However, in previous studies showing diagnostic biomarker potential of nectin-2 and nectin-4; the argument was not firmly made that nectin-2 and -4 could be therapeutic targets, or predicting prognostic and clinical properties of the disease.[10,12] With this study, we aimed to define whether overexpression of nectin proteins and mRNA remnants in plasma correlates with related clinical properties of patients with breast cancer.

Materials and Methods

Study Population and Design

The study group included 60 breast cancer patients who were receiving treatment at the Istanbul University, Institute of Oncology, between 2017 and 2018. All patients were diagnosed pathologically or radiologically. The disease was staged by physician according to the American Joint Committee on Cancer staging system. All volunteers are informed and then signed consent form for the study and ethical approval was obtained from Istanbul Medical Faculty Ethical Committee.

Venous blood samples were obtained from patients before any treatment was given, then clotted for 10 min before centrifuged. The collected serum samples stored at -20°C until analysis after the centrifugation (10 min 4000 rpm) at the room temperature.

Evaluation of Serum Nectin-2 and Nectin-4 Levels

Serum nectin-2 and nectin-4 protein levels were determined by enzyme-linked immunosorbent assay (ELISA) method (Shanghai Sunred Biological Technology Co. Ltd.). Serum samples/standards, biotinylated Fab monoclonal capture antibody, and streptavidin horseradish peroxidase conjugates were, respectively, added to the wells which are pre-coated antibody. During the 1 h incubation at 37°C, the antigen-antibody complexes formed. After this incubation, the unbound material was washed away and the colorless chromogen solution was added and again incubated at 37°C for 10 min (protect from light) for the conversion of the colorless solution to a blue solution. Since the enzyme interacts with its substrate, a color reaction occurred in direct proportion to the antigen concentration in the sample. This color reaction was stopped by the addition of an acidic stop solution and the blue color turned yellow. The colored reaction product was measured using an automated ELISA reader (ChroMate® 4300) at 450 nm. The concentrations of the samples were determined with the help of the standard curve drawn with standards of known concentration and the results were expressed as ng/L.

Quantification of Nectin-2 and Nectin-4 mRNA Expression in Serum

Nectin-2- and nectin-4-specific mRNAs in serum samples of patients' total RNA were isolated using a monophasic phenol and guanidine thiocyanate solution. Two hundred microliters of sample, 800 µl of RNA isolation solution, and 200 µl chloroform are mixed. The mixture is incubated on ice for 5 min and centrifuged at 11,000 rpm for 15 min at 4°C. The RNA phase is transferred to a 550 µl propanol-containing tube. After centrifugation at 11,000 rpm for 10 min at 4°C, RNA is washed with 75% alcohol, dried at room temperature, and transfused in 20 µl RNase-free water. cDNA synthesis was performed according to the procedure of a commercial kit (Roche, Mannheim, Germany). cDNA provides superior components that ensure total RNA templates. Nectin-2 and nectin-4 gene expression in serum were measured semi-quantitatively using GAPDH (housekeeping gen) and SYBR Green. Real-time polymerase chain reaction

(RT-PCR) process was performed using LightCycler 480 Instrument. RT-PCR components and conditions which have been used are explained in Table 1a and 1b. Probe library was used in primer selection. The confirmation of the cycle product for each molecule was carried out with melting curve analysis. Instrument measurable threshold value of fluorescence level in RT-PCR reaction the cycle it passes is called Ct (cycle threshold). According to the Ct value obtained, 2-ΔΔCt method is used.

Statistical Analysis

SPSS software was used for recording and analyzing the data (SPSS-21, Chicago, IL, USA). The Mann-Whitney U-test was applied by examining the conformity of variables to a normal distribution using visual and analytical methods (Kolmogorov–Smirnov/Shapiro-Wilk tests). P<0.05 was accepted as statistically significant.

Results

The median age of the study population was 53 (range: 24–71) years. Twenty-eight patients were in the premenopausal period. The tumor was located on the right side in 27 patients. The mean tumor size was 30.21±17.32 mm. Tumor pathology in 44 patients was pathologically reported as invasive ductal carcinoma. The number of patients with Grade 3 was 32. De novo distant metastasis was detected in four patients. Forty-one patients were in the luminal group. Lymph node involvement was detected in 25 patients. The clinical and pathological characteristics of patients are summarized in Table 2.

When the menopausal status and tumor localization of the patients included in the study were compared, no significant difference was found between the nectin-2 and nectin-4 levels. When the pathological characteristics of the patients were compared, the patients who were showed poor prognostic disease characteristics such as larger tumor size, lymph node positivity, the presence of lymphovascular invasion, presence of necrosis, and histological Grade 3 have higher serum levels of nectin protein levels than the data showing good prognosis; however, statistically insignificant. The nectin-4 expression level was statistically significantly higher in those with Ki-67 ≥30 and those with positive distant metastasis compared to the other group. In addition, nectin-2 expression was higher in patients with Grade 3 tumors which also found statistically significant. The results of nectin level measurements are shown in Table 3.

Table 1a. Real-Time PCR Components

Component name	Sample volume
Qpcr GreenMaster	10 µl
Primer F	0.6 µl
Primer R	0.6 µl
cDNA	3 µl
dH_2O	5.8 µl
Total	20 µl

PCR: Polymerase chain reaction; cDNA: Complementary DNA; dH_2O: Distilled water

Table 1b. Real-Time PCR Conditions

Condition	Temperature (°C)	Time
Polymerase Activation	95	2 minutes
Denaturation	95	15 seconds
Annealing 55	20 seconds	
Extension 72	30 seconds	

Table 2 The clinical and pathological characteristics of patients

Characteristics	n (%)
Median age (years)	53 (Range: 24-71)
Menapousal status	
Premenapouse	28 (46.7)
Postmenapouse	32 (53.3)
Tumor size (mm)	30.21±17.32
Tumor localization	
Right	27 (45)
Left	33 (55)
Histology	
Invasive ductal ca	44 (73.3)
Other	16 (26.7)
Histological grade	
Grades I–II	28 (46.7)
Grade III	32 (53.3)
Metastasis	
Absent	56 (93.3)
Present	4 (6.7)
Nodal status	
Positive	25 (41.6)
Negative	35 (58.4)
Molecular subtype	
Luminal	41 (68.3)
Non luminal	19 (31.7)

Discussion

In this study, increased serum mRNA expression of nectin-2 and nectin-4 has been detected in patients with breast carcinoma which have pathological worse

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Table 3 Distribution of Nectin-2 and 4 levels

	Nectin-2 protein	p	Nectin-2 expression	p protein	Nectin-4	p	Nectin-4 expression	p
Menapousal status								
Premenapouse	12.96±14.90	0.993	0.91±0.90	0.770	11.29±14.13	0.898	0.55±0.75	0.380
Postmenapouse	10.09±12.38		1.18±1.36		9.75±12.73		0.71±1.05	
Localisation								
Right	14.18±15.12	0.078	0.82±0.81	0.521	10.75±13.65	0.876	0.65±0.85	0.627
Left	9.25±12.04		1.24±1.36		10.33±13.31		0.60±0.96	
Lymphovascular invasion								
Yes	11.65±13.55	0.683	1.29±1.30	0.446	11.94±14.33	0.471	0.63±0.82	0.860
No	10.52±12.50		0.84±1.04		10.44±13.37		0.58±0.95	
Necrosis								
Yes	14.26±14.71	0.695	0.84±0.70	0.811	15.31±15.73	0.669	0.69±0.88	0.759
No	9.05±10.67		1.17±1.24		7.51±10.44		0.09±0.07	
Tumor size								
<2 cm	9.39±11.02	0.597	0.57±0.38	0.220	7.32±10.51	0.605	0.57±0.85	0.619
>2 cm	12.24±14.46		1.20±1.28		11.60±14.12		0.78±1.08	
Metastasis								
Yes	12.19±13.99	0.099	1.09±1.18	0.372	11.15±13.70	0.272	0.67±0.92	0.046
No	3.61±1.60		0.44±0.16		2.98±1.35		0.08±0.09	
Molecular subtype								
Luminal	13.49±14.68	0.113	1.04±1.15	0.565	12.39±14.41	0.219	0.70±0.97	0.526
Non luminal	7.11±9.96		1.05±1.19		6.32±9.67		0.46±0.71	
Pathology								
Invasive Ductal	8.90±10.35	0.165	0.85±1.00	0.074	9.10±11.80	0.324	0.65±0.98	0.192
Other	20.28±19.34		1.70±1.41		15.27±17.31		0.53±0.61	
Grade								
1 and 2	9.47±11.94	0.053	0.90±1.06	0.047	8.34±11.35	0.063	0.60±0.99	0.217
3	14.81±15.72		1.27±1.28		13.98±15.65		0.69±0.80	
Ki67								
≥30	14.53±15.86	0.139	1.07±1.23	0.831	13.80±15.38	0.109	0.82±0.93	0.019
<30	8.35±10.49		1.00±1.07		7.22±10.61		0.29±0.31	
Lenf node status								
Positive	14.20±15.79	0.212	1.07±1.09	0.895	12.55±15.19	0.336	0.59±0.86	0.822
Negative	9.41±11.51		1.02±1.21		8.92±11.70		0.65±0.95	

characteristics. Nectin-2 mRNA expression level is elevated in Grade 3 tumors. Nectin-4 mRNA expression level is elevated in metastatic patients and patients with high pathological Ki-67 levels. However, we could not able to show any statistically significant difference between serum protein levels and clinicopathologic properties of the disease. Using nectin-4 for the active follow-up of breast cancer patients might help to early detection of metastatic patients. Until now, there is no established biomarker to use for active follow-up of breast cancer patients. Current guidelines do not recommend ordering serum analysis of any biomarker even for CA-15-3. This study could be a starting point to research the usefulness of nectin molecules as a biomarker for breast cancer surveillance.

The loss of function of tight connections that control cell-cell adhesion and intracellular permeability causes the spread of cancer cells and metastasis. Nectins, which are calcium-independent immunoglobulin-like cell adhesion molecules, are in a relationship with cadherin in various intercellular associations, sometimes independently, and sometimes in cooperation with the afadine molecule.[13,14] All nectin molecules except nectin-4 are normally expressed in adult epithelial, endothelial, hematopoietic, and neuronal tissues. Although nectin-4 is expressed during embryogenesis, not detectable in adult tissues or serum.[15] Nectin-2-mediated cell adhesion has been implicated in the formation of cadherin-induced adherence complexes and the formation of a claudin bound tight linkage complex

in epithelial cells. Overexpression of nectins is related to cancer and there is evidence that tight links are necessary to cell growth regulation.

Overexpression of nectin-2 and nectin-4 in cancerous cells has been detected and has been found associated with poor prognosis in previous reports. Nectin-2 is a less frequently studied subgroup of the nectins in cancer patients. Diagnostic and prognostic value of nectin-2 in colorectal cancer,[16] pancreatic cancer,[17] gallbladder carcinoma,[18] esophageal cancer,[19] and lung cancer[20] has been studied earlier. Until now, only Oshima et al.[11] evaluated nectin-2 on ovarian cancer and breast cancer tissues pathologically using gene expression profile analysis and immunohistochemistry. They also evaluated various in vitro cancer cell cultures and found elevated levels of nectin-2. They hypothesized nectin-2 might be a treatment target since anti-nectin-2 antibodies have resulted in antigen-dependent cell death.[11] However, ensuing studies have not evolved in this era. In our study, nectin-2 is evaluated in serum and found to be related to Grade 3 tumors. Although this study requires validation, nectin-2 might be evaluated for prognostic and treatment predictive biomarker in further researches based on this pilot study. There is evidence that nectin-2 may serve as new immunogenic therapies. T-cell immunoglobulin and ITIM domain (TIGIT) recognize nectin-like adhesion molecules, specifically nectin-2, and therefore play a critical role in the innate immune response to malignant transformation.[21]

High expression of nectin-4 in ductal breast, lung, and pancreatic cancers and its relation with a poor prognosis has been shown.[9,22] Moreover, according to another study with ovarian carcinoma patients, the expression of nectin-4 could be related to resistance to chemotherapeutic agents.[23] Lattanzio et al.[22] studied nectin-4 expression in their cohort of node-negative early breast cancer. The presence of nectin-4 on cell membrane of tumor cells was associated with poor metastasis-free survival in patients with luminal-A tumors. The high serum level of nectin-4 in metastatic patients in our study suggests that it can be used as a serum marker to detect early metastasis in patients by contributing to this study. In addition, we have found that the patients with higher Ki-67 level show high nectin-4 level that also correlates with the metastatic predisposition of the tumor.

There are several limitations to this study that must be mentioned. First of all, this is a cross-sectional study that the survival results of the patients were not noted. Furthermore, there is not a normal control group, and the study cohort is categorized by medians of the patients' nectin-2 and nectin-4 serum levels. On the other hand, the main strength of the study is the comprehensive evaluation of serum nectin-2 and nectin-4 levels with two different methods; RT-PCR and ELISA. Furthermore, although tissue studies are available in the literature with this biomarker, synchronous serum analyses of these two markers in breast cancer are rare.

Conclusion

Nectin-4 mRNA is overexpressed in the patients with high Ki-67 levels and metastasis. Furthermore, nectin-2 mRNA is higher in the serum of patients with Grade 3 tumors. Further studies are recommended to determine the impact of nectin-2 and nectin-4 as a surveillance marker. Studies that are designed prospectively which contain serial measurement of serum levels of these biomarkers might give appropriate results to change the practice.

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