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Association of Rac1 Expression with Trastuzumab Resistance in HER2-Positive Breast Cancer

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ABSTRACT

Several molecular mechanisms are believed to take role in the development of trastuzumab resistance in breast cancer patients with overexpressing HER2. However, the sequence and the activity of these mechanisms are still unclear. In this study, Rac1 and neuregulin 1 (NRG1), two of ErbB pathway related proteins, were analyzed in HER2-positive breast cancer patients to investigate their roles in trastuzumab resistance. Trastuzumab resistance in metastatic breast cancer treatment was defined as a progression within six months of the treatment and in the adjuvant manner as an occurrence of local/distant recurrence before completion of treatment. Expression of Rac1 and NRG1 by immunohistochemistry (IHC) was studied in all 22 (n=12 adjuvant, n=10 metastatic) trastuzumab-resistant and 27 control tissue samples. The staining intensity of Rac1 was statistically significant in adjuvant treatment resistant group when compared with the controls (p= 0.02). In addition, when all resistant groups were compared with the control groups, Rac1 staining intensity was denser (p= 0.051). NRG1 staining intensity was in tendency to be denser as compared to control group, however it did not reach to a statistically significant level (p= 0.09). In HER2-positive breast cancer, presence of Rac1 protein is significantly associated with early response failure to adjuvant trastuzumab therapy. However, further studies with larger groups are warranted to show the value of these molecules in predicting the response to trastuzumab-based therapies.

Keywords: Breast Cancer, Neuregulin 1, Rac1, Trastuzumab resistance

ÖZET

HER2-Pozitif Meme Kanseri Hastalarında Rac1 Ekspresyonunun Trastuzumab Direnci İle İlişkisi

HER2'yi aşırı eksprese eden meme kanseri hastalarında birçok moleküler mekanizmanın trastuzumab direnci ile ilişkili olduğu düşünülmektedir. Fakat bu mekanizmaların ne kadar aktif olduğu ve hangi sıra ile etki ettiği bilinmemektedir. Bu çalışmada erbB yolağında görevli olan Rac1 and neuregulin 1 (NRG1) adlı proteinlerin HER2-pozitif meme kanserli hastalarda trastuzumab direnci ile ilişkisi araştırılmıştır. Trastuzumab direnci, metastatik meme kanserli hastalarda trastuzumab tedavisinin ilk altı ayında gelişen progresyon olarak tanımlanırken, adjuvan trastuzumab tedavisi alanlarda tedavi tamamlanmadan gelişen lokal nüks veya uzak metastaz olarak tanımlanmıştır. Rac1 ve NRG1 ekspresyonu imünohistokimyasal olarak 22 (n=12 adjuvan, n=10 metastatik) trastuzumab dirençli ve 27 kontrol hasta dokusunda çalışılmıştır. Rac1, adjuvan trastuzumab dirençli grupta kontrol grubuna göre istatistiksel anlamlı olarak daha yoğun boyanmıştır (p= 0.02). Ayrıca Rac1 açısından tüm dirençli grupla kontrol grubu karşılaştırıldığında, Rac1'in dirençli grupta daha yoğun boyandığı izlenmiştir (p= 0.051). Kontrol grubuna kıyasla dirençli grupta NRG1'in de daha yoğun boyanmasına rağmen, bu fark istatistiksel anlamlılığa ulaşmamıştır (p= 0.09). HER2-pozitif meme kanseri hastalarından adjuvan trastuzumab tedavisi alanlarda, Rac1 pozitifliği erken hastalık nüksü ile ilişkili bulunmuştur. Bu moleküllerin trastuzumab bazlı tedavilerin etkinliği ile olan ilişkisinin aydınlatılması için daha büyük çalışmalara ihiyaç vardır.

Anahtar Kelimeler: Meme kanseri, Neuregulin 1, Rac1, Trastuzumab direnci

INTRODUCTION

Approximately 20% to 25% of invasive breast cancers exhibit overexpression of the human epidermal growth factor receptor 2 (HER2, ErbB2). Overexpression of HER2 in breast cancer is associated with shorter disease-free survival (DFS), and overall survival (OS). Trastuzumab is a recombinant, humanized, monoclonal antibody directed against an extracellular region of HER2.[1] Trastuzumab is active both as a single agent and in combination with chemotherapy. The objective response rates to trastuzumab monotherapy range from 12% to 34%. Therefore, most of the HER2-overexpressing tumors demonstrate primary (de novo) resistance to trastuzumab monotherapy.[2] However, several trials have revealed that combining trastuzumab with cytotoxic agents increase response rates, time to disease progression, and survival compared with single-agent trastuzumab therapy.[3] But it is of note to mention that, some of the patients who achieve an initial response to trastuzumab-based regimens may also develop resistance within one year, which is known as secondary (acquired) resistance. Just like in the metastatic disease, trastuzumab administration in combination with or following chemotherapy improves the DFS and OS in the adjuvant setting and thus trastuzumab is, now, a cornerstone for the treatment of HER2 positive disease.[4] However, approximately 15% of the patients still develop metastatic disease despite trastuzumab-based adjuvant chemotherapy, pointing to a primary resistance to trastuzumab.[5] Therefore, the possible molecular mechanisms underlying in the primary or secondary trastuzumab resistance still needs to be elucidated.[6]

Several molecular mechanisms contribute to the development of trastuzumab resistance, including increased signaling via the phosphatidylinositol 3-kinase/Akt pathway.[7] It could activate multiple receptor pathways, for example HER2-related receptors or non-HER receptors such as the insulin-like growth factor 1.[8] Additionally, when the tumor suppressor PTEN gene and the negative regulator of Akt lose its function, it may lead to enhanced Akt signaling that causes decreased sensitivity to trastuzumab.[9] There are also other mechanisms concerning the HER2 extracellular domain (ECD) and its truncated membrane-associated fragment p95. High serum levels of HER2/ECD correlate with a poor prognosis, and the potentially enhanced signaling activity of HER2 p95 offers a possible explanation for this finding.[10] However, still there are many questions to be answered in this challenging area.

Rac1, is a member of the Rho family GTPases which belong to the Ras superfamily of small GTP-binding proteins that serve as molecular switches. Members of this superfamily appear to regulate a diverse array of cellular events, including the control of cell growth and the activation of protein kinases. It is becoming more evident that the activities of the Rho family GTPases contribute to resistance to chemotherapy.[11] Rac1 has been implicated in the downstream signaling of ErbB receptors and has been reported to be involved in the regulation of metastasis and invasion of breast cancer.[12] Increased activity of Rac1 has been shown to be related with trastuzumab resistance in different cellular studies.[13,14]

NRG1 is one of four proteins in the neuregulin family that act on the ErbB family of receptors. NRG1 encodes ligands that bind to the ErbB3 and ErbB4 tyrosine kinase receptors, while no direct high-affinity ligand for ErbB2 has been described. NRG1 binding results in ligand-stimulated tyrosine phosphorylation and activation of the ErbB receptors.[15] Several in vitro and animal studies suggest that NRG1 levels and HER3 status could help to predict tumor responsiveness to HER2-targeting therapies.[16] Besides, NRG1 can act in an autocrine or paracrine manner where autocrine NRG1 expression is involved in tumor growth and progression. This correlates with a more malignant phenotype of tumor cells. Xenograft tumors with acquired trastuzumab resistance also show overexpression of NRG1.[17]

The purpose of this study was to evaluate if there is any prominent interaction between trastuzumab resistance, either in adjuvant or metastatic setting, and these two constitutional molecules.

MATERIALS AND METHODS

Clinical Samples and Definition of Trastuzumab Resistance

We retrospectively reviewed the medical records of all breast cancer patients referred to the Medical

Oncology Department of the Ege University Faculty of Medicine. We determined the patients having HER2 (3+) by immunohistochemistry (IHC) or fluorescence in situ hybridization positivity. Age, date of diagnosis, tumor localization, histological type, stage of the disease, date and site of metastasis, and all subsequent treatments (surgery, chemotherapy, radiation therapy) were recorded from the medical report files. All patients provided written informed consent.

Trastuzumab resistance in metastatic breast cancer treatment was defined as a progression within six months while continuing with a regimen containing trastuzumab. There is no consensus in the literature defining the time interval of trastuzumab resistance, so the period of six months was chosen according to the median response duration of trastuzumab.[18] In the adjuvant treatment, trastuzumab resistance was accepted as the development of distant or local recurrence within the time of 12-month adjuvant therapy. Although the optimal duration of adjuvant trastuzumab therapy is controversial, current data and treatment guidelines support the usage for one year.[19]

Immunohistochemical Method

The study was performed using surgical or biopsy specimens of cancerous breast tissue and the expression of Rac1 and NRG1 was examined by IHC in samples of the primary tumor. All tissues were fixed in 10% buffered formalin, processed, and then embedded in paraffin. From each block, 5 μm-thick sections were cut on coated slides and dried overnight at 37^{0}C. The sections were deparaffinized in xylene, rehydrated through graded concentrations of ethanol to distilled water, and boiled in a citrate buffer (pH= 6.0) in a microwave oven for 20 minutes. IHC stainings were performed by using commercial Abcam Kits [Rabbit polyclonal IgG directed against Rac1 (Anti-Rac1 antibody ab33186) and NRG1 (Anti-NRG1 antibody ab2369)]. Blocking serum was applied for 15 minutes followed by overnight incubation with the diluted polyclonal primary antibody.

Immunohistochemical analysis was performed by a special breast cancer pathologist and manually assessed using an IHC scoring for both staining intensity and percentage. Staining intensity was assessed as: none (0), weak (1), and strong (2).

Statistical Analysis

The SPSS packet program 11.0 was used for statistical analysis. A non-parametric Mann-Whitney test was performed for statistical evaluation with $p< 0.05$ considered to be significant. Descriptive statistics of all available variables are given as numbers while percentages and results are expressed as means (SD). The selection of clinically important cut-off scores for Rac1 and Nrg1 expression was calculated by the Youden Index method based on receiver operating characteristic (ROC) curve analysis with an estimation of the variable's sensitivity and specificity.[20] The level having the closest distance to the point with both maximum sensitivity

Table 1. Demographic data and therapy properties of the patients who had either adjuvant or metastatic trastuzumab treatment

Characteristics	Resistant Adjuvant	Control Adjuvant	Resistant Metastatic	Control Metastatic
Number of patients	12	15	10	12
Age	54 ± 9	52 ± 6	50 ± 9	49 ± 14
Premenopausal	2	5	3	5
Postmenopausal	10	10	7	7
Total mastectomy	9	9	7	4
Partial mastectomy or biopsy	3	6	3	8
Adjuvant radiotherapy	12	11	4	3
Adjuvant or neoadjuvant chemotherapy	11	15	6	3

Table 2. Relation of staging, ER/PR status and pathological subtypes of the patients with Rac1 and NRG1

		RAC1 % Mean	NRG1 % Std. Dev.	Mean	Std. Dev.
TNM STAGE	1A	0,20	0,10	0,45	0,07
	2A	0,49	0,16	0,60	0,33
	2B	0,60	-	1,00	-
	3A	0,60	0,14	0,43	0,12
	3B	1,00	0,00	0,70	0,42
	3C	0,13	0,06	0,45	0,31
	4	0,41	0,18	0,51	0,23
HISTOLOGY	IDK	0,42	0,20	0,54	0,26
	Inflammatory	0,64	0,34	0,48	0,31
	IDK+Others	0,35	0,35	0,50	0,37
	Others	0,20	0,14	0,50	-
ER STATUS	0	0,51	0,24	0,50	0,28
	+1	0,27	0,21	0,63	0,21
	+2	0,25	0,07	0,50	0,00
	+3	0,30	0,15	0,70	0,26
PR STATUS	0	0,44	0,22	0,46	0,24
	+1	0,40	0,52	0,83	0,29
	+2	0,45	0,07	0,70	0,26
	+3	0,42	0,21	0,63	0,25

and specificity was assessed as the optimal cut-off level. Then, we tried to determine the confidential interval by an empirical interaction method using figures 5% below and above the cut-off values.

RESULTS

Patient Characteristics

Among the breast cancer patients that were treated in our medical oncology department, in metastatic setting 10 patients had trastuzumab resistance whereas 12 patients were resistant while on adjuvant treatment, according to the resistance definitions from above. Thus, these 22 patients were included in the analysis. As a control, fifteen patients were included in the adjuvant group who had completed their adjuvant trastuzumab therapy without any distant or local recurrence and 12 patients were included in the metastatic group who had not had any progression within the six months of therapy containing trastuzumab. The mean age of all patients was 51 ± 9 years. Demographic data of the patients and treatment details are shown in Table 1.

There are seventeen (77.2%) patients with negative estrogen receptors in total of the adjuvant and metastatic groups who are resistant to trastuzumab treatment. In the control groups, sixteen (59.2%) patients had estrogen receptor negativity. Most (n= 14, 63.6%) of these breast cancer patients with trastuzumab resistance had invasive ductal carcinoma of the breast. Four of them had inflammatory carcinoma, two had both invasive ductal and lobular carcinoma, and two had other pathological subtypes. All patients in the control groups had invasive ductal carcinoma with the exception of four patients who had inflammatory carcinoma and other pathologies. Relation of staging, ER/PR status and pathological subtypes of the patients with Rac1 and NRG1 are shown in Table 2.

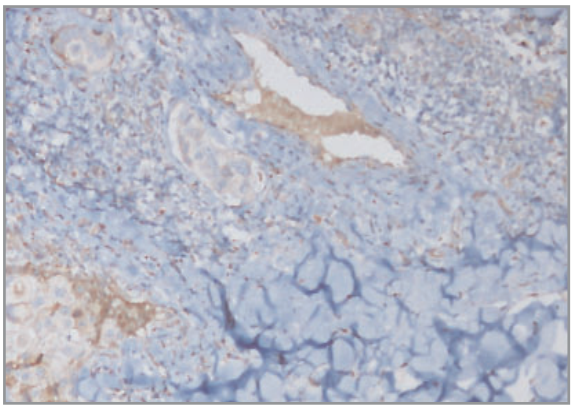

Figure 1. Figure mostly points negative staining of cytoplasmic Rac1 and only in few parts points +1 Rac1 positivity (arrow) at X20 high power magnification in a patient of control group who had not trastuzumab resistance in the adjuvant treatment.

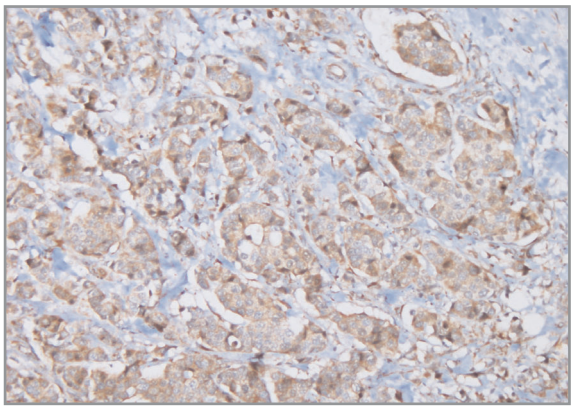

Figure 2. Figure points intense staining of cytoplasmic Rac1 positivity at X20 high power magnification in a patient who had trastuzumab resistance in the adjuvant treatment.

Immunohistochemical Results

When we compared our immunohistochemical scores of Rac1 and NRG1 in the trastuzumab resistant groups with their controls, we found that Rac1 staining was denser in the trastuzumab resistant breast cancer group, but only for adjuvant treatment receivers (p= 0.02). There were eight patients with +2, and two patients with +1 staining intensity of Rac1 in the resistant group and this was statistically denser as compared to control group (Figure 1, 2). However, NRG1 staining intensity, although was in tendency to be denser as compared to control group, did not reach to a statistically significant level (p= 0.09). There were five (41.6%) patients in the trastuzumab-resistant adjuvant group having +2 staining intensity for NRG1 whereas there were no patients in the control group and NRG1 was negative in only two patients for resistant adjuvant group, however, in contrast to this, there were seven patients having no NRG1 staining in the control group (Table 3). In addition, when we compared all the resistant groups (adjuvant and metastatic) (n= 22) with the control groups (n= 27), the presence of Rac1 also showed a borderline importance statistically, in favor of the resistant groups (p= 0.051).

Then, we evaluated the staining ratios of these proteins in order to accomplish a possible cut-off value for the resistance in the adjuvant treatment. We used ROC curves so as to determine a cut-off value for our clinical test. Staining intensity for Rac-1 at 55% and for NRG1 at 75% was found to be the optimal cut-off values according to this method. However, it is important to expand our data's sensitivity and specificity. Therefore, to control and confirm our results, we calculated the confidential interval by an empirical interaction method. We evaluated 5% below and above the optimal cut-off values for each protein. This application showed us that intervals of 50-55% for Rac1 and 50-95% for NRG1 were also acceptable as cut-off levels. The huge difference between the upper and lower limit of NRG1 interval decreased its sensitivity while the selected cut-off interval that we found for Rac1 appear to have a good discriminating power to differentiate the presence of protein from controls.

DISCUSSION

HER2 overexpression is associated with more aggressive tumor behavior and poor prognosis in breast cancer.[21] Although trastuzumab considerably improves the outcome for HER2-positive breast cancer patients, unfortunately, not all patients benefit from that treatment. A fraction (20%) of early stage breast cancer patients will reoccur despite trastuzumab treatment and 70% of patients with metastatic disease who receive trastuzumab monotherapy will become resistant to treatment.[22] Thus, to elucidate some critical molecules related to trastuzumab resistance is one of the important problems that need quick solutions in daily oncologic practice.

Table 3. Number of the patients in the control and trastuzumab-resistant groups according to IHC staining intensity of Rac1 and NRG1

Staining Intensity	Resistant Adjuvant (n)	Control Adjuvant (n)	Resistant Metastatic (n)	Control Metastatic (n)
Rac1 negative	2	5	2	5
+1 Rac1staining	2	5	4	3
+2 Rac1staining	8	5	4	4
NRG1 negative	2	7	3	2
+1 NRG1 staining	5	8	6	8
+2 NRG1 staining	5	0	1	2

Rac1, a Ras-like small GTPase, has been implicated in the control of cell growth and morphology. Overexpression of Rac1 has been reported in breast cancers while its activity was in close relationship with ErbB signaling. Increased activity of Rac1 was believed to be associated with breast cancer invasion, progression and metastasis.[23,24] Dukmanovic et al. showed that a specific Rac1 inhibitor reduced Rac1 activity in trastuzumab-resistant SKBR3 cells. This inhibitor restored trastuzumab-mediated endocytic down-regulation of ErbB2 and decreased extracellular signal-regulated kinase activity in resistant cells. So, they found out the important role of Rac1 in trastuzumab resistant human breast cancer cells.[13] It has also been indicated that the association of Rac1 with ErbB2 leads to prolonged activation of Rac1. This seems to be mediated by TGF-ß as it modulates HER2 signaling to Rac1-Pak1 by compartmentalizing a complex to the cell. When active in cancer cells, this mechanism would lead to metastatic progression.[14] However, Rac1 becomes activated only upon phosphorylation. Thus, the presence of Rac1 may relate to trastuzumab resistance only if it gets activated through upstream kinases as the activated Rac1 is able to form a stable complex with HER2. In addition, data from pre-clinical studies reveal that, up-regulation of Rac1 activity in HER2 overexpressing cells may be blocked by trastuzumab treatment. This suggests that the initial escape from the trastuzumab-mediated inhibition of Rac1 activity may be an important mechanism leading to the high levels of Rac1 activity. Therefore, this may describe the undesirable relationship between increased Rac1 activity and trastuzumab resistance.[25] These findings also support our data that the more Rac1 protein is present, the more the resistance of breast cancer is seen, especially in the adjuvant setting.

NRG1 seems likely to play a role in epithelial cancers since it encodes ligands that bind to the ErbB3 and ErbB4 tyrosine kinase receptors which concomitantly recruit ErbB1 and ErbB2 coreceptors as heterodimers. This results in ligand-stimulated tyrosine phosphorylation and activation of the ErbB receptors. These ligands, originally known as the heregulins alpha and beta, neu differentiation factor, and glial growth factor II are made by alternative splicing. They include forms that are transmembrane, intracellular, externally membrane-bound, shed, or secreted.[26] Although the NRG1-encoded proteins are usually thought of as mitogens, they can also be powerfully pro-apoptotic. Particularly, expressing NRG1 in cells can cause apoptosis of these expressing cells.[27] NRG1 expressions have not been widely studied in HER2-positive breast cancer cell lines, but in clinical samples, HER2-positive tumors express NRG1 less frequently than the HER2 negative tumors.[28] Detectable levels were found in two HER2-positive cell lines and showed considerably high expression levels. In addition, exogenous NRG1 has been shown to induce trastuzumab resistance on the trastuzumab-sensitive cell lines.[29] Although, we have not been able to show a linkage between NRG1 protein presence and trastuzumab resistance, this is possible due to the small number of patients in our study, an important limitation of our study.

However, despite some limitations, we believe that the findings presented in our study are interesting in the light of studies highlighting the role of Rac1 and NRG1 in HER2 signaling. In the literature, there are studies implicating that Rac1 and NRG1 were related to resistance to trastuzumab treatment. Their results suggested that trastuzumab resistance is significantly dependent on Rac1 and NRG1 activity through an impaired activation of Erb signaling, and for HER2-amplified breast cancer patients without any Rac1 and NRG1 expression, trastuzumab is likely to be more effective.[12,15] So, our findings confirmed that especially, the Rac1 protein levels may strongly suggest the prediction of tumor response to trastuzumab. It is highly likely that an overexpression of Rac1 could identify a subgroup of HER2-positive tumors with a high activity of proliferation and survival pathways along with a resistance to trastuzumab.

For evaluating IHC results, not only staining intensity, but also staining percentage is also very important. It should improve the clinical utility of the IHC findings and cut_off scores which are generally in use for determining the positivity of biomarkers.[30] In the literature, there is not an accepted cut-off value for Rac1 in terms of staining percentage. For this reason, in order to assess an optimal cut-off level, we used the ROC curve analysis and found it to be 55%. However, further validation of this cut-off score is needed to be repeated in order to accept it as a clinically relevant threshold.

The issue of trastuzumab resistance is becoming increasingly important as recent trials strongly support a role for trastuzumab in both the adjuvant and metastatic setting for ErbB2 overexpressing breast cancer.[3,4] Thus, it is urgent to find out the molecular pathways that contribute to trastuzumab resistance. Once the proteins in the ErbB receptor pathway are clarified, novel therapeutic targets can be identified and improvements in overall survival can be accomplished.[2,5] Rac1 and NRG1 activity may provide a predictive marker for acquired trastuzumab resistance of ErbB2 overexpressing breast cancers, and agents targeting these molecules may be used in treatment. A better understanding of these findings, however, may require further investigation in clinical trials with larger patient population.

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