ERBB2 juxtamembrane domain (trastuzumab binding site) gene mutation is a rare event in invasive breast cancers overexpressing the *ERBB2* gene

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The recent development of targeted therapies using monoclonal antibodies has added new dimensions to breast cancer treatment. Trastuzumab has been added to the regimens that contain chemotherapeutic agents, which has improved the clinical outcomes of patients in both the adjuvant and metastatic settings. However, trastuzumab resistance, both *de novo* and acquired, continues to be problematic. There have been scattered studies reporting *ERBB2* gene mutation, but nothing is currently known about the *ERBB2* binding site mutations. In the current study, we examined the *ERBB2* juxtamembrane domain trastuzumab binding site for mutations in invasive breast cancers overexpressing *ERBB2*. Pure tumor cells of 54 breast cancer patients were procured using laser capture microdissection. Two polymerase chain reaction primer pairs were designed to amplify the trastuzumab binding site sequence. The polymerase chain reaction product was sequenced. Standard clinicopathological data were recorded. For the 54 patients, there was one (2%) case that showed missense point mutation in exon 17 (H559A). There were nine patients treated with trastuzumab in the metastatic setting, none of which had gene mutation. Therefore, we conclude that *ERBB2* juxtamembrane domain (trastuzumab binding site) gene mutation is a rare event in breast cancer. Although it is unclear whether this substitution would result in trastuzumab target therapy resistance, this would not account for the relatively high frequency of this resistance encountered clinically.

Modern Pathology (2011) 24, 1055–1059; doi:10.1038/modpathol.2011.64; published online 15 April 2011

Keywords: breast cancer; gene mutation; ERBB2; trastuzumab

Overexpression of the tyrosine kinase receptor ERBB2 (HER2) occurs in approximately 18–20% of early-stage invasive breast cancers.^{1,2} The ERBB2 receptor protein has an extracellular domain, a transmembrane domain, and an intracellular tyrosine kinase domain.³ Phosphorylation of key residues and the recruitment of cytoplasmic signaling molecules that subsequently initiate signaling

cascades occur through dimerization of the ERB receptors.

Increasingly, management decisions regarding whether oncologists should prescribe adjuvant treatment are being based on the biology of the patient's tumor, with a consideration of the hormone receptor status,⁴ and more recently, with the assessment of ERBB2.⁵ The identification of the amplification of *ERBB2* gene and the overexpression of its protein product has resulted in targeted monoclonal antibody-based therapy.^{6,7} Preclinical studies indicate synergistic anti-tumor activity when trastuzumab is combined with a number of anti-cancer drugs. Additive cytotoxic interactions between trastuzumab and other agents, including paclitaxel,

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Received 11 October 2010; revised 17 February 2011; accepted 18 February 2011; published online 15 April 2011

also have been shown.⁸ However, several patients who initially received trastuzumab in the adjuvant setting develop recurrent tumor and resistance within 1 year in the metastatic setting.⁹ This resistance has become a major clinical concern. There is no clinically verified factor that can be used to predict trastuzumab resistance. Several molecular mechanisms contributing to trastuzumab resistance have been proposed.¹⁰

The crystal structure of the ERBB2 receptor by itself and in combination with trastuzumab was deduced. The receptor has an extracellular region composed of 630 amino acids, a single membrane-spanning region and a cytoplasmic tyrosine kinase. The extracellular region is composed of four domains. These four domains are arranged as tandem repeats of a two-domain unit consisting of an L domain (domains I and III; ~190 amino acids each) and a cysteine-rich domain (domains II and IV; ~120 amino acids each). Trastuzumab binds to ERBB2 receptor to a region in domain IV that is occupied by the domain II finger-like projection in other HER receptors. By crystallography, the antigen-antibody interaction is mediated by three regions along the C-terminal portion of domain IV. The first region is a loop formed by amino acid residues 557–561, the second from residues 570–573, and the third from residues 593–603. The coding region of the first two residues is present in Exon 17 and the third residue is present in Exon 18. The antigen-antibody binding interactions formed by the first and third loop are mainly electrostatic, whereas the second is predominantly hydrophobic.¹¹ The ability of trastuzumab to bind to this region correlates to its efficacy. It is postulated that bound antibody may achieve its therapeutic effect through several mechanisms, which may include, but are not limited to, antibody-dependent cell-mediated cytotoxicity, G1 arrest, receptor down-regulation, or the prevention of heterodimer formation.^{12–14} However, to be efficacious, the therapeutic antibody must be able to bind to the receptor. The binding of an antibody to an antigen is entirely dependent upon non-covalent interactions. Small changes in the antigen structure can profoundly affect the strength of the antibody-antigen interaction. The loss of a single hydrogen bond at the interface can reduce the strength of interaction over a 1000-fold. Hence, changes in the *ERBB2* sequence can potentially affect the binding efficacy of trastuzumab.

The structure of the ERBB2 receptor and its relationship to trastuzumab theoretically may have a role in *de novo* or acquired resistance. No study to date has tested the antibody interacting binding site of the juxtamembrane domain for possible mutations and correlated them with clinical evidence of resistance or recurrence in humans. The intent of this study is to analyze the critical regions of the *ERBB2* juxtamembrane domain trastuzumab binding site for mutations.

Materials and methods

Cases Selection

Patients diagnosed with invasive breast cancer that overexpressed ERBB2, detected by fluoresce *in situ* hybridization, were identified in Roswell Park Cancer Institute during the time frame of 1997–2004. Tissue samples from these patients were present in the form of archival, formalin-fixed paraffin-embedded material. There were 556 invasive breast cancer cases. ERBB2 overexpression was demonstrated by fluoresce *in situ* hybridization in 132 (24%) cases. Microscopic evaluation of tissue sections demonstrated enough tumor cells for laser capture microdissection in 106 cases. Polymerase chain reaction rendered enough extracted DNA identified through spectrophotometry using NanoDrop in 54 (53%) cases.

Laser Capture Microdissection, Polymerase Chain Reaction, and Sequencing

An appropriate slide and block from the identified cases are selected. Five-micron thick sections are cut and placed onto a slide, deparaffinized, and lightly stained with hematoxylin and eosin, followed by dehydration. Tumor cells were selectively procured from the slide by an Arcturus PixCell II LCM. At least 2000 pulses with size of 10 μ were applied. The tumor cells were then lysed and the DNA isolated by a commercially available spin column kit, designed specifically for small quantities (PinPoint Isolation kit, Zymo Research). Polymerase chain reaction was performed using primers that encompass the nucleotides that correspond to amino-acid residues 557–561, 570–573, and 593–603. As the DNA is being isolated from formalin-fixed tissue, which is notorious for degrading DNA into small fragments of <300 bp, and as the distance between amino-acid residue 557 and 603 correspond to over 900 bp, two primer pairs were designed. One primer pair encompassed the nucleotides corresponding to amino-acid residues 557–561 and 570–573 (forward primer 5'-CCGAGTACTGCAGGGGTATG-3'; reverse primer 5'-CTCACCGGTCCAAAACAGGT-3') and the other primer pair encompassed the nucleotides corresponding to amino acid residues 593–603 (forward primer 5'-ACAAAGGGGACCCAACTAA-3'; reverse primer 5'-GGCATGTAGGAGAGGTCA GG-3'). The former primer pair produced a 192-bp amplicon, whereas the latter primer pair produced a 186-bp amplicon. Polymerase chain reaction for both primer pairs utilized a modified hot start at 95°C for 2 min followed by 35 cycles with denaturation at 95°C for 15 s, annealing at 54°C for 15 s, and extension at 72°C for 30 s. A final extension phase at 72°C for 10 min ended the reaction. A small amount of the PCR product was then visualized on an ethidium bromide-stained 2% gel in 100 V field. All amplicons were sequenced using Big Dye Terminator Sequencing (ABI PRISM 7700).

Results

Patients Characteristics

Patients' age ranged from 35 to 90 years (median 58). There were 45 (83%) ductal, 4 (7.5%) lobular, 4 (7.5%) mixed ductal and lobular, and 1 (2%) micropapillary subtypes. The tumor was located in the right breast in 23 (43%) cases, left breast in 30 (55%) cases, and bilaterally in one (2%) case. Modified Scarff Bloom Richardson grading was grade I in 5 (9%), grade II in 16 (30%), and grade III in 33 (61%) cases. Lymph nodes (LN) metastasis was absent in 25 (46%) cases (clinically in 10, and pathologically in 15 cases). Lymph nodes metastasis was identified in 1 to 3 LNs in 11 (20%) cases, 4 to 9 LNs in 10 (19%) cases, and >9 LNs in 8 (15%) cases. Tumor size was recorded in all cases but one. It was ≤1 cm in 33 (62%) cases, 1.1–2 cm in 16 (30%) cases, and >2 cm in 4 (8%) cases. Ductal carcinoma *in situ* was absent in 11 (20%) cases, present in ≤50% of tumor size in 36 (67%) cases, and >50% of tumor size in 7 (13%) cases. Hormonal receptors including estrogen receptor and progesterone receptor were recorded. Although hormonal receptor was absent in 22 (41%) for ER and 31 (57%) for PR, both hormones were absent simultaneously in 21 (39%) cases. Local and/or distal metastasis was developed in 13 of 42 (31%) cases. After tumor recurrence, trastuzumab therapy was provided for 10 patients. Therapy was discontinued in one patient because of therapy-related side effects (Table 1).

ERBB2 Binding-Site Sequencing Results

One of 54 (2%) cases showed missense point mutation coding amino-acid residue 559, substituting histidine by arginine amino acid at residue 559 (H559A).

Clinical History of the Patient with Mutation

The patient is 76-year-old postmenopausal female with high-grade triple-positive (estrogen receptor/progesterone receptor/ERBB2) ductal carcinoma, measuring 1.1 cm and with negative lymph nodes. Postoperatively, she received a course of radiation therapy, followed by a 5-year course of oral tamoxifen. No trastuzumab was given. The patient was disease-free after 5-year follow-up.

Discussion

There is no clinically verified factor that can be used to predict trastuzumab resistance in breast cancer. There are many proposed mechanisms of resistance including reduction of antibody affinity, and binding due to MUC4 overexpression was related to trastuzumab resistance in cell lines.^{15,16} Downstream signaling pathway members were proposed

Table 1 Clinicopathological characteristics of 54 cases

Variable	Results
Age, range (median) (in years)	35–90 (58)
Tumor type (total)	54
Ductal, <i>n</i> (%)	45 (83)
Lobular, <i>n</i> (%)	4 (7.5)
Mixed lobular and ductal, <i>n</i> (%)	4 (7.5)
Micropapillary, <i>n</i> (%)	1 (2)
Laterality (total)	54
Right, <i>n</i> (%)	23 (43)
Left, <i>n</i> (%)	30 (56)
Bilateral, <i>n</i> (%)	1 (2)
SBR grade (total)	54
I, <i>n</i> (%)	5 (9)
II, <i>n</i> (%)	16 (30)
III, <i>n</i> (%)	33 (61)
Lymph node metastasis (total)	54
N0, <i>n</i> (%)	25 (46) ^a
N1, <i>n</i> (%)	11 (20)
N2, <i>n</i> (%)	10 (19)
N3, <i>n</i> (%)	8 (15)
Size (total)	53 ^b
T1a, <i>n</i> (%)	3 (6)
T1b, <i>n</i> (%)	7 (13)
T1c, <i>n</i> (%)	23 (43)
Total T1, <i>n</i> (%)	33 (62)
T2, <i>n</i> (%)	16 (30)
T3, <i>n</i> (%)	4 (8)
DCIS (total)	54
0%, <i>n</i> (%)	11 (20)
1–50%, <i>n</i> (%)	36 (67)
>50%, <i>n</i> (%)	7 (13)
ER (total)	54
0%, <i>n</i> (%)	22 (41)
1–50%, <i>n</i> (%)	8 (15)
51–100%, <i>n</i> (%)	24 (44)
PR (total)	54
0%, <i>n</i> (%)	31 (57)
1–50%, <i>n</i> (%)	13 (24)
51–100%, <i>n</i> (%)	10 (19)
ER/PR (total)	54
Both 0%, <i>n</i> (%)	21 (39)
At least one positive, <i>n</i> (%)	33 (61)
Distant metastasis	50 ^c
M0, <i>n</i> (%)	38 (76)
M1, <i>n</i> (%)	12 (24)
Trastuzumab therapy	10 ^d
With response, <i>n</i> (%)	6 (67)
With no response, <i>n</i> (%)	3 (33)

^aLN metastasis was absent clinically in 10 cases and pathologically in 15 cases.

^bSize not documented in one case.

^cIn four cases, evaluation for local or distant metastasis was not available.

^dOne patient did not tolerate therapy.

as potential source for trastuzumab resistance, including p27 Kip1, PTEN, PI3K, mTOR, and Akt.^{17–19} Cross-talk with other signaling pathways including insulin-like growth factor receptor-1, ER pathway, and vascular endothelial growth factor that have the ability to bypass ERBB2 blockade have also been proposed to be involved in trastuzumab resistance.^{20–22} However, these studies were all preclinical or in cell lines, and to date, there are no clinically validated markers to predict *de novo* or

acquired resistance to ERBB2-targeted therapy. We have recently proposed a gene signature that could predict trastuzumab resistance and worse clinical outcome.²³ However, another possibility could be the binding site of the juxtamembrane domain.

Previous studies have searched for *ERBB2* mutations in breast tumors and cell lines using a candidate mutation approach, sequencing of pooled amplicons, or sequencing of individual *ERBB2* alleles from tumors with gene amplification. Stephens *et al*²⁴ sequenced the complete *ERBB2* coding sequence in 173 tumors, 18 of which were breast, and the kinase domain in 303 cancers (56 breast) and 235 cell lines (9 breast, none of those sequenced in this report). No mutations were found in any of the breast tumors or cell lines, and amplification status in the breast tumors and cell lines was not indicated. Lee *et al*²⁵ used single-stranded conformation polymorphism to look for mutations in exons 18–23, which encode for the ERBB2 kinase domain, in 378 tumors of breast, colorectal, and gastric. Mutations were found in 4.3, 2.9, and 5% of cases, respectively. However, none of breast cancer cases had *ERBB2* amplification. Gori S *et al*²⁶ have sequenced exons 19–22, which encode the kinase domain of 41 patients with metastatic breast carcinoma and treated with trastuzumab. Two insertions in exon 20 (P780-H781insC) and one missense mutation in exon 21 (S856P) were found. Zito CI *et al*²⁷ have sequenced the whole *ERBB2* gene in four breast cancer cell lines and in one ovarian cell line. They found deletion in the coding region of exon 5 and 4 non-syn substitutions, one in exon 7 (C295X), two in exon 17 (I655V), and one in exon 22 (A890S).

None of these studies had specifically targeted the binding site of trastuzumab. Given the discovery of the crystal structure of ERBB2 receptor, illustrating the binding site with trastuzumab¹¹ and the mystery of trastuzumab resistance, we thought of specifically targeting the trastuzumab binding site, looking for possible mutation that could explain this resistance.

In our previous report, we documented presence of mutation in 20 of 54 cases using temperature gradient capillary electrophoresis.²⁸ However, sequencing these cases along with temperature gradient capillary electrophoresis-negative cases showed only one missense mutation. This mutation had resulted in substituting histidine by arginine amino acid at residue 559 (H559A). Arginine and histidine share some properties, including being both hydrophobic and charged. However, they differ in the side chain, van der Waals volume (148 vs 118), and the later being aromatic. It is unclear whether these differences make any significant change in the binding properties of the ERBB2 receptor and trastuzumab, or modify the function of the ERBB2 receptor. It would be important to define the exact nature of mutation, and later investigate the ERBB2 protein and trastuzumab interaction, through possibly protein crystallography. However,

the only patient who carries this mutation did well, with at least 5-year disease-free without trastuzumab therapy.

In the studied cases, only patients who developed tumor recurrence were treated with trastuzumab. Overall, 13 patients had tumor recurrence, 9 of which had been treated with trastuzumab. None of these patients had binding site gene mutation. Therefore, we conclude that even if a binding site gene mutation exists and is consistently repetitive and not merely due to chance, and truly results in trastuzumab resistance, it cannot be accounted for the high frequency of trastuzumab resistance.

We conclude that a greater understanding of the biological heterogeneity within the ERBB2 positive cohort of breast cancer patients, particularly related to trastuzumab resistance is needed. Our data suggests that gene mutations in the *ERBB2* juxtamembrane domain (trastuzumab binding site) is a rare event in breast cancer and is unlikely to account for the relatively high frequency of therapeutic resistance encountered clinically. Further studies that explore the relationship between tumor biology and response to adjuvant cytotoxic chemotherapy and ERBB2-targeted therapy are needed to help with the selection of optimal treatment regimens. We think that the proper direction for exploring the biology of trastuzumab resistance would be looking for a specific predictive gene signature.^{23,29}

Acknowledgement

We thank the Alpha Foundation for the financial support.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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