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HER-2 PROTEIN EXPRESSION AND ITS PROGNOSTIC VALUE IN BREAST CANCER – OVERVIEW OF EXPERIENCE AT THE UNIVERSITY HOSPITAL FOR TUMORS, ZAGREB, CROATIA

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Summary

Immunohistochemical analysis of the HER-2 protein expression in ductal invasive breast cancer was done on archival paraffin-embedded breast cancer tissue using specific monoclonal antibodies. All of the 190 analyzed patients were treated at the University Hospital for Tumors, Zagreb, Croatia, from September 2002 to September 2003. Year of birth, tumor size, histological type of tumor, histological grade, lymph-node status, steroid receptors, HER-2 expression, nuclear grade and vascular invasion were determined for each patient. HER-2 overexpression was found in 24% of the patients. HER-2 overexpression was not associated with age, but it was associated significantly with tumor size, worse histological and nuclear grades, lack of steroid receptors, lymph node involvement and positive vascular invasion. Steroid receptors expression was associated significantly with better nuclear grade and negative vascular invasion.

KEYWORDS: *breast cancer, HER-2, immunohistochemical analysis*

IZRAŽAJNOST PROTEINA HER-2 I NJEGOVA PROGNOSTIČKA VRIJEDNOST U KARCINOMU DOJKE

Sažetak

Izražajnost HER-2 proteina te steroidnih receptora u duktalnom invazivnom karcinomu dojke određena je imunohistokemijski, uporabom monoklonskih protutijela. Korišten je arhivski bioptički materijal uklopljen u parafinske blokove. Svih 190 analiziranih bolesnica liječeno je u Klinici za tumore, Zagreb, Hrvatska, u razdoblju od rujna 2002. do rujna 2003. godine. Svakoj bolesnici utvrđena je godina rođenja, veličina tumora, histološka slika tumora, histološki gradus tumora, zahvaćenost pazušnih limfnih čvorova metastazama, nalaz steroidnih receptora, nalaz HER-2 proteina, nuklearni gradus te vaskularna invazija. Analizom imunohistokemijskih rezultata utvrđena je izražajnost HER-2 proteina kod 24% bolesnica. Nije utvrđena statistički značajna razlika u izražajnosti HER-2 proteina s obzirom na dob bolesnica, a utvrđena je povezanost HER-2 proteina s nepovoljnijim prognostičkim čimbenicima: većom veličinom tumora, nepovoljnijim stupnjem diferenciranosti tumora, većom zahvaćenosti pazušnih limfnih čvorova metastazama, negativnim nalazom steroidnih receptora, pozitivnom vaskularnom invazijom te nepovoljnijim nuklearnim gradusom. Statistički je utvrđena i povezanost steroidnih receptora s povoljnijim prognostičkim čimbenicima: povoljnijim nuklearnim gradusom i negativnom vaskularnom invazijom.

KLJUČNE RIJEČI: *rak dojke, HER-2, imunohistokemijska analiza*

INTRODUCTION

The human epidermal growth factor receptor 2 (HER-2)/neu (c-erbB-2) gene is located on chromosome 17q 21-22 and encodes a transmembrane tyrosine kinase receptor protein (HER-2 protein) that is a member of the epidermal growth factor receptor (EGFR) or HER family.

HER-2 protein is placed at normal and tumor breast cells, with increased expression at tumor breast cells. HER-2 protein is overexpressed in breast cancer, ovarian cancer, endometrial cancer, lung cancer, gastric cancer, etc. (1, 2). HER-2 protein overexpression has been identified in 20-30% of invasive breast cancers (3, 4).

HER-2 protein is a prognostic and predictive factor in breast cancer (5). HER-2 overexpression is associated with shorter overall survival, worse histological and nuclear grade, lack of steroid receptors, p53 mutation, etc. (prognostic value) (3, 6-10). HER-2 protein overexpression is a predictive factor of response to immunotherapy (trastuzumab, lapatinib), chemotherapy (anthracyclines), hormonal therapy (aromatase inhibitor) and local relapses in patients undergoing surgery or radiation therapy (11-28). Testing of newly diagnosed breast cancer specimens for HER-2/neu status has now become a "standard of practice".

HER-2 protein is a transmembrane tyrosine kinase receptor which has tyrosine kinase activity and is involved in the process known as signal transduction, in which external growth factors, or ligands, affect the transcription of various genes by phosphorylating a series of transmembrane proteins and intracellular signalling intermediates.

HER-2 protein is a member of EGFR or HER family together with HER-1, HER-3 and HER-4. While no known ligand for HER-2 protein has been identified, unlike other HER family members, the signalling pathway is carried out through heterodimerization (dimerization with a different member of the family) (28-31).

Breast cancer has humoral and cellular response to HER-2 protein (32). Its sequence (IISAVVGIL) together with HLA-A2 molecule stimulates *in vitro* T lymphocyte reaction. Animal immunization with that peptide leads to cytotoxic and helper T lymphocyte reaction and the production of specific antibodies.

The assessment of HER-2 status can be performed using various techniques, such as IHC (immunohistochemistry), FISH (fluorescence in situ hybridization), CISH (chromogenic in situ hybridization) or RT-PCR (reverse transcriptase-polymerase chain reaction). IHC assesses HER-2 protein overexpression, while other techniques assess HER-2/*neu* gene amplification. IHC and FISH are the predominant methods (33-40).

The FDA (Food and Drug Administration) has approved two commercially available IHC kits and one of them (the HercepTest™) has been used in this study. Lately, circulating serum HER-2 protein level and phosphorylated HER-2 are being determined as prognostic and predictive factors in breast cancer (41-43).

Trastuzumab (Herceptin), a monoclonal humanized antibody, was developed to specifically bind the extracellular portion of HER-2 receptor. The FDA has approved trastuzumab as a treatment for advanced metastatic disease and in adjuvant treatment for earlier stage disease. Trastuzumab is currently studied in neoadjuvant treatment protocols (44-50). Trastuzumab is approved for patients with IHC +++ or FISH +. The patients with IHC ++ must undergo FISH (51-54).

Lapatinib (Tykerb) is a small molecule, dual tyrosine kinase inhibitor (TKI) of EGFR (HER-1) and HER-2, that enters the cell and blocks the function of this and other proteins. The FDA has approved lapatinib in combination with another cancer drug, capecitabine (Xeloda), for patients with advanced, metastatic breast cancer that is HER-2 positive. The combination is indicated for women who have received prior therapy with other cancer drugs, including an anthracycline, a taxane and trastuzumab (28).

The aim of this study was:

1. To determine the possibility of IHC assessment of HER-2 protein overexpression in ductal invasive breast cancer
2. To determine HER-2 protein association with other breast cancer prognostic factors (tumor size, histological grade, steroid receptors, lymph node involvement, vascular invasion, nuclear grade)
3. To determine HER-2 protein association with age (patients younger and older than 50),

with the intention to discover another breast cancer prognostic factor.

Namely, breast cancer biological behavior is still unknown and there are still cases which do not respond to applied therapy. Therefore, any additional indicator is helpful to improve therapy moving towards tailored treatment for individual patients.

PATIENTS AND METHODS

In this prospective study, archival biopsy material from the Department of Clinical Pathology, University Hospital for Tumors, Zagreb, Croatia, was used. Material from 190 patients, operated for ductal invasive breast cancer from September 2002 to September 2003, was analyzed. Resected material was treated with standard pathohistological techniques, icluding tissue fixation in 10% formalin, paraffin-embedded tissue, tissue cutting to thickness from 3 µm to 5 µm and hemalaun eosin painting.

Histological diagnosis was then made according to Bloom-Richardson histological grade (well differentiated, moderately differentiated and poorly differentiated). Three morphologic features were used and scored ranging from 1 to 3, as follows:

1. The degree of tumor tubule formation	Score
>75% tubules	1
50-75% tubules	2
<50% tubules	3
2. The nuclear pleomorphism of tumor cells	**Score**
little, normal, uniform	1
moderate polymorphism	2
expressed polymorphism	3
3. The mitotic activity of the tumor (rate of cell division)	**Score**
0-10	1
11-20	2
21-30	3

The scores were then added together for a final sum ranging between 3 to 9. This value was then used to grade the tumor as follows:

Grade 1 tumor (well differentiated)	3-5
Grade 2 tumor (moderately differentiated)	6-7
Grade 3 tumor (poorly differentiated)	8-9

For each tumor sample, size of the tumor, histological and nuclear grade, vascular invasion and axillary lymph node status were determined.

According to lymph nodes findings, the patients were divided into 3 groups:

1. Patients with negative lymph nodes
2. Patients with 1-3 positive lymph nodes
3. Patients with 4> positive lymph nodes.

According to age, patients were divided into 2 groups:

1. Patients younger than 50 (premenopausal)
2. Patients older than 50 (postmenopausal).

Additional sections, three from each tumor, were used for immunohistochemistry testing of steroid receptors and HER-2 protein overexpression in the tumor sample.

Steroid receptors, estrogen and progesterone receptors (ER and PR), were assessed by means of monoclonal antibodies. For estrogen receptors, a mouse monoclonal antibody ready to use DAKO; N^0 H 7098 was used, and for progesterone receptors, a mouse monoclonal antibody DAKO; N^0 M 3569; 1:50. The immunohistochemistry assessment of steroid receptors was performed using a semiquantitative method as follows:

1. Negative reaction (0) – less than 5% of tumor cells show positive nuclear reaction
2. Poorly positive reaction (+) – 5-10% of tumor cells show positive nuclear reaction
3. Intermediate high reaction (++) – 11-50% of tumor cells show positive nuclear reaction
4. High positive reaction (+++) – >50% of tumor cells show positive nuclear reaction.

The immunohistochemistry assessment of HER-2 protein overexpression was performed by means of the HercepTest™ (kit DAKO N^0 K 5204). The results were presented according to the manufacturer's reference as follows:

1. Negative reaction (-) – no staining is observed, or membrane staining is observed in <10% of tumor cells
2. Negative reaction (1+) – a faint/barely perceptible membrane staining is detected in >10% of tumor cells. The cells exhibit incomplete membrane staining.
3. Weakly positive (2+) – a weak to moderate complete membrane staining is observed in >10% of tumor cells
4. Strongly positive (3+) – a strong membrane staining is observed in >10% of tumor cells.

Statistical analysis was done using χ^2 test with a security level at $p<0.05$.

RESULTS

Immunohistochemical analysis of the HER-2 protein overexpression was done in 190 patients with breast carcinoma, operated from September 2002 to September 2003. HER-2 protein overexpression was found in 46 patients or 24% (Table 1).

Table 1.

PATIENT NUMBER

HER-2 positive	46
HER-2 negative	144
Overall	190

According to age, patients were divided into 2 groups (Table 2): 1) patients younger than 50 (premenopausal), 2) patients older than 50 (postmenopausal). There was no statistically significant difference in HER-2 overexpression between the groups (p=0.732).

Table 2.

HER-2 PROTEIN OVEREXPRESSION AND PATIENT AGE

Patient group	Patient No
HER-2 pos. <50 yrs	13
HER-2 neg. <50 yrs	35
HER-2 pos. >50 yrs	33
HER-2 neg. >50 yrs	109
Overall	190

HER-2 protein overexpression was significantly associated with other breast cancer prognostic factors: tumor size, worse histological grade, lymph node involvement, lack of steroid receptors, positive vascular invasion, worse nuclear grade.

The tumor size was determined postoperatively and expressed in centimeters. According to the tumor size, patients were divided into 3 groups (Table 3): patients with tumor <2 cm (T1), patients with tumor of 2-5 cm (T2), patients with tumor >5 cm (T3). There were statistically significant differences in HER-2 protein overexpression in patients with T2 tumor in relation to patients with T1 tumor (p=0.008), and in patients with T3 tumor in relation to T1 tumor (p=0.039). There was no statistically significant difference in HER-2 protein overexpression in patients with T2 tumor in relation to patients with T3 tumor (p=0.868).

Table 3.

HER-2 PROTEIN OVEREXPRESSION AND TUMOR SIZE

Patient group	Patient No
HER-2 pos. T1	15
HER-2 neg. T1	85
HER-2 pos. T2	24
HER-2 neg. T2	48
HER-2 pos. T3	7
HER-2 neg. T3	11
Overall	190

According to the histological grade, patients were divided into 3 groups (Table 4): patients with grade I (GR I) tumor (well differentiated tumor), patients with grade II (GR II) tumor (moderately differentiated tumor) and patients with grade III (GR III) tumor (poorly differentiated tumor). There was a statistically significant difference in HER-2 protein overexpression in patients with GR II tumor in relation to patients with GR I tumor ($p<0.001$), and in patients with GR III tumor in relation to patients with GR I tumor ($P<0.001$). There was no statistically significant difference in HER-2 overexpression in patients with GR II tumor in relation to patients with GR III tumor (p=0.181).

Table 4.

HER-2 PROTEIN OVEREXPRESSION AND HISTOLOGICAL GRADE

Patient group	Patient No
HER-2 pos. GR I	3
HER-2 neg. GR I	58
HER-2 pos. GR II	22
HER-2 neg. GR II	56
HER-2 pos. GR III	21
HER-2 neg. GR III	30
Overall	190

According to lymph node involvement, patients were divided into 3 groups: patients with negative lymph nodes, patients with 1-3 positive lymph nodes and patients with 4>positive lymph nodes (Table 5). There were statistically significant differences in HER-2 overexpression in patients with 4> positive lymph nodes in relation to pa-

Table 5.

HER-2 PROTEIN OVEREXPRESSION AND LYMPH NODE INVOLVEMENT

Patient group	Patient No
HER-2 pos. LN 0	16
HER-2 neg. LN 0	87
HER-2 pos. LN 1-3	10
HER-2 neg. LN 1-3	36
HER-2 pos. LN 4>	20
HER-2 neg. LN 4>	21
Overall	190

tients with negative lymph nodes (p<0.001) and to patients with 1-3 positive lymph nodes (p=0.015). There was no statistically significant difference in HER-2 overexpression in patients with negative lymph nodes in relation to patients with 1-3 positive lymph nodes (p=0.491).

According to steroid receptors status, patients were divided into 2 main groups: patients with positive steroid receptors (R+) and patients with negative steroid receptors (R-). Patients with R+ include patients with ER+PR+, ER+PR- and ER-PR+, while R- patients are ER-PR- patients. There was statistically significant difference in HER-2 overexpression in R- patients in relation to R+ patients (p<0.004, Table 6). According to ER and PR status, patients were divided into 8 subgroups (Table 7). There were statistically significant differences in HER-2 overexpression in ER-PR- patients in relation to ER+PR+ patients (p<0.001), in ER+PR- patients in relation to ER+ PR+ patients (p=0.019) and in ER-PR+ patients in relation to ER+PR+ patients (p=0.021). In other subgroups, statistically significant differences in HER-2 overexpression were not determined.

According to the finding of vascular invasion, patients were divided to 2 groups: patients

Table 6.

HER-2 PROTEIN OVEREXPRESSION AND STEROID RECEPTORS

Patient group	Patient No
HER-2 pos. SR+	27
HER-2 neg. SR+	117
HER-2 pos. SR-	19
HER-2 neg. SR-	27
Overall	190

Table 7.

HER-2 PROTEIN OVEREXPRESSION AND ESTROGEN AND PROGESTERONE RECEPTORS

Patient group	Patient No
HER-2 pos. ER+PR+	4
HER-2 neg. ER+PR+	51
HER-2 pos. ER+PR-	8
HER-2 neg. ER+PR-	21
HER-2 pos. ER-PR+	15
HER-2 neg. ER-PR+	45
HER-2 pos. ER-PR-	19
HER-2 neg. ER-PR-	27
Overall	190

with positive vascular invasion and patients with negative vascular invasion (Table 8). There was a statistically significant difference in HER-2 overexpression in patients with positive vascular invasion in relation to the patients with negative vascular invasion (p<0.001).

Table 8.

HER-2 PROTEIN OVEREXPRESSION AND VASCULAR INVASION

Patient group	Patient No
HER-2 pos. VI pos.	15
HER-2 neg. VI pos.	8
HER-2 pos. VI neg.	31
HER-2 neg. VI neg.	136
Overall	190

According to the nuclear grade (NG), patients were divided into 3 groups (Table 9): patients with nuclear grade I (NG I) tumor (well differentiated tumor), patients with nuclear grade II (NG II) tumor (moderately differentiated tumor) and pa-

Table 9.

HER-2 PROTEIN OVEREXPRESSION AND NUCLEAR GRADE

Patient group	Patient No
HER-2 pos. NG I	8
HER-2 neg. NG I	89
HER-2 pos. NG II	32
HER-2 neg. NG II	41
HER-2 pos. NG III	6
HER-2 neg. NG III	14
Overall	190

tients with nuclear grade III (NG III) tumor (poorly differentiated tumor). There was a statistically significant difference in HER-2 protein overexpression in patients with NG II tumor in relation to patients with NG I tumor ($p<0.001$) and in patients with NG III tumor in relation to patients with NG I tumor ($P=0.019$). There was no statistically significant difference in HER-2 overexpression in patients with NG II tumor in relation to patients with NG III tumor ($p=0.391$).

Besides HER-2 protein overexpression, steroid receptors expression in relation to nuclear grade and vascular invasion was determined.

Steroid receptor expression in relation to nuclear grade is seen in Table 10. Patients were divided into 3 groups: patients with NG I, patients with NG II and patients with NG III. There was a statistically significant difference in steroid receptor expression in patients with NG I tumor in relation to patients with NG II tumor ($p<0.001$) and in relation to patients with NG IIII tumor ($p<0.001$). There was no statistically significant difference in HER-2 overexpression in patients with NG II tumor in relation to patients with NG III tumor ($p=0.293$).

Table 10.
STEROID RECEPTORS EXPRESSION AND NUCLEAR GRADE

Patient group	Patient No
ER+PR+ NG I	43
ER+PR- NG I	16
ER-PR+ NG I	29
ER-PR- NG I	9
ER+PR+ NG II	12
ER+PR- NG II	9
ER-PR+ NG II	24
ER-PR- NG II	28
ER+PR+ NG III	0
ER+PR- NG III	4
ER-PR+ NG III	7
ER-PR- NG III	9
Overall	190

According to the finding of vascular invasion, patients were divided to 2 groups: patients with positive vascular invasion and patients with negative vascular invasion (Table 11). There was statistically significant difference in SR expression in patients with negative vascular invasion in relation to patients with positive vascular invasion ($p=0.002$).

Table 11.
STEROID RECEPTORS EXPRESSION AND VASCULAR INVASION

Patient group	Patient No
ER+PR+ VI pos.	2
ER+PR- VI pos.	3
ER-PR+ VI pos.	10
ER-PR- VI pos.	8
ER+PR+ VI neg.	53
ER+PR- VI neg.	26
ER-PR+ VI neg.	50
ER-PR- VI neg.	38
Overall	190

DISCUSSION AND CONCLUSION

HER-2 protein overexpression has been identified in 20%-30% of invasive breast cancers (3, 4).

HER-2 protein is a prognostic and predictive factor in breast cancer (5). HER-2 overexpression is associated with shorter overall survival, worse histological and nuclear grade, lack of steroid receptors, p53 mutation, etc (negative prognostic value) (3, 6-10). HER-2 protein overexpression is a predictive factor of response to immunotherapy, chemotherapy, hormonal therapy and local relapses in patients undergoing surgery or radiation therapy (11-28).

This study has determined IHC assessment of HER-2 protein overexpression in ductal invasive breast cancer, HER-2 protein association with other breast cancer prognostic factors (tumor size, histological grade, steroid receptors, lymph node involvement, vascular invasion, nuclear grade) and HER-2 protein association with age (patients younger and older than 50). The immunohistochemistry assessment of HER-2 protein was performed by means of the HercepTest™ and in the positive group (3+), there were patients with a strong membrane staining in >10% of tumor cells. Out of 190 patients operated for invasive breast cancer, HER-2 protein overexpression was found in 24% of them, which is in accordance with other results (from 20% to 30%) (3, 4). HER-2/*neu* gene amplification with FISH or CISH was unfortu-

nately not performed in our clinic during 2002 and 2003. If it had ben performed, the number of HER-2 positive patients would probably be somewhat bigger, but still in accordance with other results.

According to age, there was no statistically significant difference in HER-2 protein overexpression between premenopausal and postmenopausal patients. Literature data differ, but most of them show HER-2 protein overexpression in young patients (<35 years) and some data show equal HER-2 protein expression according to age (55, 56).

Correlation with the tumor size and HER-2 protein overexpression could also be found in literature database (57). In this study, statistically significant differences in HER-2 protein overexpression in patients with T2 and T3 tumor in relation to patients with T1 tumor were determined. This confirms association of HER-2 protein with the tumor size which is a negative prognostic factor.

HER-2 protein overexpression was also associated with a worse histological grade, with GR II and GR III, which is corresponds with data found by other authors (56-58).

HER-2 protein overexpression was also determined with other unfavorable breast cancer prognostic factors. It was significantly associated with lymph node involvement (4>LN), lack of steroid receptors, positive vascular invasion and worse nuclear grade (NG II and NG III). All results are in line with data by other authors (56-60).

Steroid receptors expression in relation to the nuclear grade and vascular invasion was also determined. Steroid receptors are positive prognostic and predictive factors in breast cancer and their expression was determined to show the difference from HER-2 protein overexpression which is a negative prognostic and predictive factor in breast cancer. Steroid receptors expression was associated significantly with a better nuclear grade (NG I) and negative vascular invasion, which clearly shows the difference in relation to HER-2 protein overexpression.

These results show the significance of HER-2 testing in patients with invasive breast cancer considering its prognostic and predictive value. At the same time, patients with IHC ++ must undergo FISH or CISH because the best response to immunotherapy is achieved with patients with IHC +++ or with FISH positive patients. Besides HER-2 testing, it is important to assess other prognostic factors in invasive breast cancer (tumor size, histological and nuclear grade, lymph node involvement, steroid receptors expression, vascular invasion) in order to identify patients with aggressive tumor type, increased risk of recurrence and decreased overall survival. Namely, all current studies directed towards discovering new prognostic factors have finally come to conclusion that classical prognostic factors (see above) are still the most important criteria for disease prognosis and that the new ones are just the additional indicators.

New studies also show the importance of assessing all HER (EGFR) family members, instead of just one, in order to get the real profile of receptor expression. The data showed correlation of EGFR (HER-1) and HER-2 with other poor prognostic factors, while c-erbB-3 (HER-3) and c-erbB-4 (HER-4) are not correlated with overall survival (61). Some studies have determined c-erbB-3 connection with the tumor size and histological grade (62, 63), while other studies have determined c-erbB-4 connection with a good histological grade and positive estrogen receptors (64-67). All these results refer to still insufficient knowledge about EGFR family members, their interaction with and influence on tumor cells.

HER-2 testing is also important for the prediction of response to immunotherapy (trastuzumab), chemotherapy (anthracyclines, taxanes) and hormonal therapy (aromatase inhibitors).

Steroid receptors determination is important for therapy determination because of their positive prognostic value and their predictive value (hormonal therapy).

According to findings obtained in this study it can be concluded:

1. Possibility of IHC assessment of HER-2 protein overexpression in ductal invasive breast cancer is determined
2. HER-2 overexpression is found in 24% of patients with invasive breast cancer
3. HER-2 protein overexpression is not associated with age
4. HER-2 protein overexpression is associated with negative breast cancer prognostic factors (tumor size, worse histological and nuclear grade, lack of steroid receptors,

lymph node involvement, positive vascular invasion)

5. Steroid reception expression is associated with positive prognostic factors (better nuclear grade and negative vascular invasion).

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