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RESEARCH ARTICLE

THE IMPACT OF NOTTINGHEM PROGNOSTIC INDEX (NPI) ON THE OCCURRENCE OF RELAPSES IN HER-2 / NEU POSITIVE BREAST CANCER

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ABSTRACT

Introduction: Immunohistochemical detection of Human epidermal growth factor (HER-2 / neu) is necessary in the evaluation of breast cancer. According to data in the literature, 10-30% of breast cancers show expression of this marker, which applies as unfavorable prognostic factor. Therefore, it is important in the selection of patients who will benefit from adequate treatment with Herceptin. Aim of this study is to determine the impact of clinical, histopathological and immunohistochemical parameters and NPI- Nottingham prognostic index on the occurrence of relapses in HER-2 / neu positive breast cancer.
Material and Methods: In this retrospective study, 174 patients were included. All of the patients were previously treated from primary breast cancer, and were analyzed in histopathological laboratory in Acibadem / Sistina clinical Hospital in the period from June 2007 to June 2010. Beside the analysis of clinical and histopathological parameters as well as the NPI, additional immunohistochemicaltests were evaluated in order to determine the HER-2 / neu status, the Ki67 proliferative index, and the expression of the protein product of the tumor suppressor gene p53. During the follow-up period (42 to 80 months) relapses were observed in 38 patients (21.8%).
Results: The age of patients ranged from 28-83 (average 55.48, $\pm$10.0) years. Lymphnode metastases were found in 99 (57%) patients. The tumor was poorly differentiated in 70 patients (40%). Immunohistochemical expression of HER-2 / neu was observed in 33 patients (19%) and relapses of the disease were present in 13 of them (40%). In 30 of the 33 HER2 / neu-positive patients (90%), NPI was higher than 3.4. Despite this association, the expression of HER-2 / neu was correlated with tumor diameter, lymph node status, mitotic index, degree of histological differentiation, lymph-vascular invasion, stage of the disease and the expression of p53 protein product and proliferative index Ki67 ($p<0.05$).
Conclusion: The score of theNottingham prognostic index higher than 3.4 which included the tumor size, lymphonodal status and degree of histological differentiation of the tumor, is an excellent parameter in evaluating the course of the disease in the HER-2 / neu positive breast cancers compared to the occurrence of relapses.

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INTRODUCTION

The incidence of breast cancer, in the world and in our country, is the most common cancer in women (Ferlay *et al*., 2013). Receptor of the Human Epidermal growth factor (HER-2 / neu or c-erbB2) is identified by the chemical carcinogen ethyl-nitroso-urea, which was induced in the rat neuroblastomas (Fehm *et al*., 2004). Human homologue of HER-2 / neu is present in the 17th chromosome and encodes a 185 kd transmembrane glycoprotein. In breast cancer, amplification of the HER-2 / neu and expression of HER-2 / neu protein is associated with a poor prognosis with an average survival of 3 years (Fehm *et al*., 2004; Chen *et al*., 2004) compared with the 6-7 year survival in HER-2 / neu negative cases. Trastuzumab (Herceptin®) is a human monoclonal antibody that selectively binds to the extracellular part of the 185 kd HER-2 / neu protein (Miles, 2001). It is the first "target" or gene therapy for breast cancer which showed a good response and improved survival. Testing the status of HER-2 / neu oncogene is introduced into routine practice in the world and is performed by immunohistochemical analysis, which gives good results considering the standardized procedures and protocols established by the American Association of Clinical Oncologists (ASCO) (Wolff *et al*., 2007), which reduces the subjective assessment of evaluation (Wolff *et al*., 2013).

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Fluorescent in situ hybridization (FISH) and other hybridization methods (conventional - CISH, hybridization with silver – SISH, etc.) represent additional sensitive and accurate methods for the quantitative evaluation of HER-2 / neu gene status, but are more expensive and require special laboratory conditions and equipment as well as more time for evaluation. Therefore, hybridization techniques are not recommended in routine practice in evaluation of HER-2 / neu status and are reserved for selected patients in which the immunohistochemicalexpression is incomplete according to the criteria of ASCO (Wolff *et al*., 2013).

The aim of this study is to determine the impact of Nottingham prognostic index on the occurrence of relapses in HER-2 / neu positive breast cancers and to distinguish important prognostic clinicopathological parameters (age, tumor diameter, lymphnode status, degree of histological differentiation, nuclear grade, mitotic index, lymph-vascular invasion, stage of disease) and immunohistochemical parameters (expression of the protein product of the tumor suppressor gene p53 and proliferative index Ki67), in this group of patients.

MATERIAL AND METHODS

An audit of 174 histopathological findings of breast cancers paatients was performed. All the patients were diagnosed in the histopathological laboratory of the Acibadem / Sistina clinical Hospital, from June 2007 to June 2010. From this group of patients,according to immunohistochemical analysis, 33 (19%) of the patients belong to the category of HER-2 / neu positive tumors. Routine sections were first analyzed with standard staining for hematoxylin and eosin. In routine breast cancer diagnostic procedures, classical histological parameters were evaluated: tumor diameter - T, lymphnode status – N, and degree of histological differentiation - G. The degree of histological differentiation is evaluated according to the modified Nottingham scoring system of Bloom-Richardson (Bloom and Richardson, 1957). Of those three parameters of the Nottingham scoring system, nuclear grade and mitotic index were evaluated separately.

These histological parameters are incorporated in performing the Nottingham prognostic index - NPI which is calculated according to the equation (0,2 x T) + N + G (Galea *et al*., 1992). In this equation the values for N are 1 = without metastatic deposits, 2 = 1-3 positive nodes and 3 = more than three positive nodes, and the values of G, 1 = well differentiated, 2 = moderately differentiated and 3 = poorly differentiated tumors. The obtained results are categorized into three prognostic groups - low-risk group with the value up to 3.4; with high-risk score from 3.4 to 5.4 and a very high risk to score over 5.4 (Galea *et al*., 1992). In our study intermediate-risk and high-risk group are allocated in one category (high-risk) in terms of low-risk group for convenience of statistical analysis. With the other routine analyzes, the age of the patients is incorporated, as well as the stage of the disease, according to a postoperative histopathological classification of malignant neoplasms of the breast of the American Joint Cancer Committee - AJCC, from 2010 (Edge *et al*., 2010). Representative samples of the tumor were selected and further processed for immunohistochemical analysis. Freshly cut sections with a thickness of 2.5 microns, were placed in special pretreated slides (Poly-l-lysine) and dried overnight at a temperature of 60^0C. They were dewaxed in xylene and dehydrated in alcohol with different concentrations (100%, 96%, 80%). Epitope retrieval of HER2 / neu receptor was used in water bath at a temperature of 96^0C, and forimmunohistochemical studies of proliferative index Ki67, and protein products of the p53 tumor suppressor gene citrate buffer was used in a microwave oven of 700W, according to the requirements of the manufacturer. Imunohistohemical analysis was performed by avidin-biotin complex technique. Evaluation of HER-2 / neu gene was performed by ready-to-use, HercepTest kit (DAKO, Glostrup, Denmark), and the for the immunohistochemical analysis of Ki67 proliferative index and the protein product of the tumor suppressor gene p53 prepared monoclonal murine antibody (DAKO, Glostrup, Denmark) and visualizing system (Dako REAL ™ EnVision ™ Detection System, Peroxidase / DAB +, Rabbit / Mouse) have been used with the dilution of 1:50. At the slides with a HER-2 / neu positive and negative controls were added for accurate evaluation and control staining. After the performed steps of immunohistochemical staining, slides were counterstained with hematoxylin. Evaluation of HER-2 / neu consists in detecting strong membrane staining in at least 10% of malignant cells in the invasive front of the tumor, which was considered as positive result and indicated with 3+ (Wolff *et al*., 2007). Evaluation of the nuclear signal for Ki67 proliferative index and the protein product of the tumor suppressor gene p53 was performed according to the rules of Bhargava (2009) and Reed (2000) by counting at least 10 high power fields (HPF, x40) which were considered as positive on 14% of nuclei of malignant cells for Ki67 and over 10% of nuclei of cancer cells for p53. The type of the antibody, clone, dilution, the manufacturer and the cut-off point of a positive signal for the tested antibodies are shown in Table 1.

Table 1. Type of the antibody, clone, dilution, manufacturer and the cut-off point of positive/negative signal

Antibody	Clone	Dilution	Manufacturer	Cut-offpoint*
HER-2/neu	-		DAKO, Glostrup, Denmark	3+ (>10%)
Ki-67	MIB1	1:50	DAKO, Glostrup, Denmark	>14% (positive)
p53	DO7	1:50	DAKO, Glostrup, Denmark	>10% (positive)

* Evaluation of Herceps test was performed according the criteria of the American Society of Clinical Oncology (ASCO). For the other two antibodies, modified proposals of Bhargava (2009) and Reed (2000) were accounted.

The categorization of the analyzed parameters were represented by numbers (percentages), and their association in respect of expression of HER-2 / neu were expressed by Pearson-'s $\chi 2$ and Fisher-'s exact test. Statistical significance was determined by the values of $p<0.05$.

RESULTS

The age of the patients was 28-83 years (mean 55.48, SD$\pm$10.07 years), and 120 patients (68%) or two thirds of the

entire group was over 50 years old. Ninety-nine (57%) of these 174 patients, at the time of diagnosis had a tumor diameter greater than 2 cm with positive lymph node status. In 116 patients (59%) NPI was higher than 3.4, that belonged to the unfavorable prognostic group of patients. High proliferative activity of the primary tumor determined by Ki67 antibody, was observed in 113 patients (65%), and in this study relapses were identified in 38 patients (21%).

The expression of HER-2 / neu oncogene was identified in 33 patients (19%). There was a positive correlation between expression of HER-2 / neu oncogene with the parameters of the postoperative histopathological classification of cancer, namely, a tumor diameter - pT (p = 0.04) and lymph node status - pN (p = 0.001) as well as and the stage of the disease (p = 0.007). There was also a strong association with the parameters of Bloom-Richardson's modified Nottingham scoring system that determine the degree of histological differentiation - G (p = 0.002), or nuclear grade - NG (p = 0,001) and mitotic index (p <0,01). All these parameters were reflected along the NPI which shows that it is more pronounced with HER-2 / neu positive breast cancers that are disadvantageous prognostic group (p = 0,001) compared with other patients. Lymph-vascular invasion was present in 30 of 33 (90%) HER-2 / neu positive breast cancers (p = 0.01).

Table 2. Clinical and histological parameters in the group of 174 patients with breast cancer related to the expression of HER-2 / neu oncogene

Parameters	No. of patients (%)	HER2+	HER2-	p-value
Age				
≤ 50 y.	54 (32)	9	45	
> 50 y.	120 (68)	24	96	0,69
Tumor size (pT)				
<2 см	75 (43)	9	66	
≥2 см	99 (57)	24	75	0,04
Lymph nodes status (pN)				
LN +	99 (57)	27	72	
LN)	75 (43)	6	69	0,001
Differentiation(G)				
G1,G2	104 (60)	12	92	
G3	70 (40)	21	49	0,002
Nucleaar grade (NG)				
NG1, NG2	128 (73)	17	111	
NG3	46 (27)	16	30	0,001
Mitotic index				
≤10 mitoses	141 (81)	9	132	
>10 mitoses	33 (19)	24	9	2,11E-18
NPI				
NPI ≤ 3,4	58 (41)	3	55	
NPI > 3,4	116 (59)	30	86	0,001
Lymph-vascular invasion				
Present	128 (73)	30	98	
Absent	46 (27)	3	43	0,01
Stage of the disease				
I+II	123 (70)	17	106	
III+IV	51 (30)	16	35	0,007
Proliferative index				
Ki67 ≤14%	61 (35)	4	57	
Ki67 >14%	113 (65)	29	84	0,002
p53				
p53 ≤ 10%	125 (71)	18	107	
p53 > 10%	49 (29)	15	34	0,01
Relapses				
Present	38 (21)	13	25	
Absent	136 (79)	20	116	0,006
Total (%)	174 (100)	33 (19)	141 (81)	

In terms of immunohistochemical parameters, the level of proliferative activity of the tumor cells determined by Ki67 antibody was more pronounced in the HER-2 / neu positive tumors (p = 0.002) as well as and the expression of the protein product of tumor suppressor gene p53 (p = 0.01). The analyzed parameters and their ratios are presented in Table 2. Regarding the present relapses, identified in 38 patients, 13 of them belongto the HER-2 / neu-positive patients (34%). Of these 13 patients, loco-regional metastases were found in 9 (70%), bone metastases in 2 (15%), and metastasis in visceral organs in 2 (15%) patients.

Legend: HER2 - human epidermal growth factorreceptor, NPI - Nottingham prognostic index; p-value refers to the parameters categorized as: the age group of the patients equal or up to 50 years, compared to the group over 50 years old, tumor status (size of the primary tumor) up to 2 cm (pT1) and more than 2 cm (pT2, pT3, pT4), LN + positive and LN- negative lymph nodes, well and intermediate differentiated tumors (G1 / G2) in relation to poorly differentiated (G3), low and intermediate nuclear differentiation (NG1 / NG2) compared the high degree of nuclear differentiation (NG3) and disease stages I and II compared to more advanced stages of the disease III and IV.

DISCUSSION

In our study, the age of the patients as a clinical parameter, did not show any association with the expression of HER-2 / neu oncogene that were confirmed in studies of Partridge (2013) and Arvold (2011). In these studies,with a large series of patients (1703 patients in the first and 1434 in the other study),the age of the HER-2 / neu positive patients is an independent prognostic factor compared with other types of breast cancer.

The size of the primary tumor in our study was proved to be an important parameter in correlation with HER-2 / neu status. As in other studies (Korkolis *et al*., 2004; Cortesi *et al*., 2013), our results showed that the HER-2 / neu positive breast cancers have larger tumor diameter, but not to the extent that was found in other molecular subgroups, such as the "triple" negative cancers cancer (Kim *et al*., 2006). Similar results were reported in a study by Michaelson and coworkers (Michaelson *et al*., 2002).

The lymph-node status is a strong prognostic parameter in all types of breast cancers and in HER-2 / neu positive breast cancers it was associated with lymph-vascular invasion as shown in our as well as in some other published studies (Slamon *et al*., 1987; Millar *et al*., 2009). Cited data in the report of Cardoso *et al*. (2001), showed thateven negative finding of the ipsilateral axillary lymph nodes, increases the risk of the recurrent disease in HER-2 / neu positive primary tumors.

The degree of histological differentiation is one of the most analyzed parameters in the evaluation of prognostic factors in breast cancer. In our series it is evaluated separately by Bloom-Richardson's modified Nottingham scoring system (Bloom and Richardson, 1957), as well as by both incorporated parameters - mitotic index and nuclear grade. In studies where these

parameters are analyzed, there is a strong association between them and the expression of HER-2 / neu oncogene was found (21,22).

Aaltomaa and colleagues (Ménard *et al*., 2001) in 1991 published a study that included 688 patients with breast cancer in which emphasize the importance of nuclear grade and mitotic index as important prognostic parameters in the evaluation of the course and outcome of breast cancer. With the introduction of new immunohistochemical and molecular biological methods of evaluation Weigel and Dowsett in 2010 suggest that these classical parameters have not lost their importance, but rather only confirm their role in the classification of favorable and unfavorable groups of patients with breast cancer (Weigel and Dowsett, 2008). In all studies that describe the association of HER-2 / neu with the occurrence of relapses (Bhargava *et al*., 2009; Slamon *et al*., 1987; Millar *et al*., 2009) mitotic index and nuclear grade incorporated in the degree of histological differentiation are important factors in the prediction of the course of the disease as was outlined in our study.

The Nottingham Prognostic Index (NPI) unites classic parameters of postoperative histopathological classification of breast - the tumor diameter, lymph-node status, and thedegree of histological differentiation of the tumor which through the Nottingham scoring system incorporate two important elements - mitotic index and the nuclear grade (Lee and Ellis, 2008). Taking into account all previously described parameters that are strongly correlated with the expression of HER-2 / neu, as it was expected, and this parameter reflects the importance of prediction of recurrent disease in HER-2 / neu positive breast cancer as it is described in our and in other studies (Ménard *et al*., 2001; Miller *et al*., 2004; Kollias *et al*., 1999). In our series, 30 out of 33 patients with HER-2 / neu positive breast cancers are in unfavorable prognostic group (90%). This result is similar to the results of Tovey (2009) that reported 68% and Chia (2008) with 73% in a series of patients that have a negative lymph-node status, up to 87% of the patients reported in the series of Sidoni and coworkers (Sidoni *et al*., 2004). The parameters that are incorporated in the postoperative histopathological classification which determine the stage of the disease, showed that they correlate with the expression of Her-2 / neu thus, the stage of the disease, is an important element in determining the course and outcome of the disease.

Lymph-vascular invasion is a phenomenon which is more prevalent in certain molecular subtypes of breast cancers and is associated with lymph-node status (Pinder *et al*., 1993; Simpson and Page, 1994) and the expression of HER-2 / neu, as it is published in some studies (Kim *et al*., 2006; Millar *et al*., 2009) and as shown results in our series. Immunohistochemical parameters reported in our series showed a positive correlation with the expression of HER-2 / neu. According to the results, as it was expected, the high mitotic index reflects the strong expression of proliferative index determined by monoclonal antibody Ki67 in this particular group of patients, especially that in most studies this parameter is treated as an independent prognostic factor regardless of the type of breast cancer (Bhargava *et al*., 2009; Reed *et al*., 2000; Millar *et al*., 2009). Expression of the protein product of the tumor suppressor gene p53, according to recent studies (Melhem-Bertrandt *et al*., 2012) is a powerful parameter which is associated with positive expression of HER-2 / neu, as published in the study of Reed and coworkers (Reed *et al*., 2000) where in a series of 613 patients was singled out as an independent prognostic parameter.

Conclusion

According to the results of our study, the classic histopathological parameters incorporated in postoperative histopathology classification of breast, along with the stage of the disease, and the parameters involved in determining the degree of histological differentiation of primary breast cancer are relevant factors that reflect the values of Nottingham Prognostic Index (NPI). Compared with the HER-2 / neu negative breast cancers, NPI was proved as excellent predictor in classifying HER-2 / neu positive breast cancer that in our series have a high rate of recurrent or metastatic disease. On the other hand, in addition of determining the prognostic groups of HER-2 / neu positive breast cancer, other immunohistochemical parameters must be taken into account that will imply adequate treatment of these patients.

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