Comparison of differential PCR and immunohistochemistry for the evaluation of c-erbB-2 in breast carcinoma and relationship to other prognostic factors

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The c-erbB-2 gene codes for a membrane receptor protein that is homologous to the epidermal growth factor receptor. Differential polymerase chain reaction (PCR) is an alternative semi-quantitative method for evaluating gene amplification and can be performed in formalin-fixed paraffin-embedded specimens. We aimed to compare differential PCR and IHC (immunohistochemistry) in the determination of c-erbB-2 status of breast cancers. Correlation between the prognostic impact of c-erbB-2 gene amplification and protein overexpression with conventional prognostic factors were also evaluated. Differential PCR and IHC for c-erbB-2 were performed on formalin-fixed paraffin sections of 60 invasive breast cancers. Results and the relation with the other prognostic parameters were compared. A highly significant degree of concordance between differential PCR and IHC in the evaluation of c-erbB-2 status of breast carcinoma was detected. Amplification and overexpression were significantly related to the number of metastatic lymph nodes, histologic grade, and lymphatic invasion but not age, histologic type, tumor size and estrogen status. We demonstrated and confirmed the importance of c-erbB-2 overexpression and amplification as a single and combined prognostic parameter together with conventional factors and confirmed that it can be detected by both immunohistochemistry and differential PCR techniques in breast carcinoma. This semi-quantitative technique provides reliable results and can be used routinely.

Key words: Breast carcinoma, c-erbB-2, prognostic parameters.

C-erbB-2 (HER-2/neu) oncogene, represents a family of normal cellular gene which is involved in cell growth and differentiation. Now it is clear that alterations either in gene structure, amplification or overexpression play a role in the pathogenesis of some human cancers like breast carcinoma. c-erbB-2 gene encodes a 185 kd transmembrane glycoprotein that has a homology to the receptor with the epidermal growth factor receptor. The amplification of c-erbB-2 gene results in elevated levels of c-erbB-2 messenger RNA and protein that can be detected by immunohistochemistry (IHC) [10, 19, 27].

Several studies evaluated the prognostic influence of c-erbB-2 amplification and overexpression in breast carcinoma. Firstly, Slamon et al showed that c-erbB-2 gene was amplified in 27% of breast carcinomas [22]. And further studies confirmed these findings [1, 3, 16, 24, 28].

There are variety of methods available to determine the c-erbB-2 status of breast cancer. Techniques to evaluate gene amplification include Southern and slot-blot testing, polymerase chain reaction (PCR) methods, *in situ* fluorescent hybridization (FISH) and nonfluorescent hybridization. Qualitative and quantitative c-erbB-2 measurements have been performed with IHC on frozen and formalin-fixed paraffin embedded tissues, Western blot testing and enzyme-linked immunosorbent assay (ELISA). Most of these assays require prospective collection of fresh tissue and are not applicable to archive material [2, 7, 15, 19, 27].

In this study we described a simple, highly sensitive, alternative differential polymerase chain reaction (DPCR) method for detecting amplification of c-erbB-2 in a routine pathology laboratory and compared it with immunohistochemical staining of c-erbB-2 protein. Differential PCR and immunohistochemical results are compared with other clinical and morphologic prognostic parameters.

Material and methods

Samples from sixty cases of female breast carcinoma from the archives of Pathology department of Cukurova

University were evaluated. Histologic slides of each case were reviewed for diagnostic reassessment. Grading was performed according to Bloom-Richardson and nuclear grade. Tumor size, number of positive lymph nodes, presence of lymphatic invasion were also evaluated. Sections of two benign breast lesions (fibrocystic change and fibroadenoma) were used as negative control for DPCR.

Immunohistochemistry for c-erbB-2 protein and estrogene receptor. Five μm thick sections of formalin-fixed, paraffin-embedded tissue samples were deparaffinized and rehydrated through a series of graded alcohols. Antigen retrieval was carried on in microwave oven with "peroxidase blocking reagent" for 8–9 minutes. The slides were kept in citrate buffer for 20 minutes at room temperature, then rinsed in PBS. Sections were incubated for 20–30 minutes with acid-urea solution, washed and then incubated 20–30 minutes with goat serum. After washing, the sections were incubated with anti c-erbB-2 (polyclonal rabbit antibody, DAKO, Denmark) and anti estrogene receptor (Novacastro, UK) at room temperature for 90 minutes, washed and incubated 30 minutes with biotinylated horse anti-mouse IgG immunglobulin (DAKO, Denmark). After washing, the sections were incubated for 30 minutes with streptavidin peroxidase reagent and washed again. The immunperoxidase was visualized with AEC (3 amino 9 ethyl carbazole), (DAKO, USA). The sections were counterstained with Mayer's hematoxylin and then coverslipped.

Evaluation of c-erbB-2 expression by IHC. Only membrane staining intensity and pattern were evaluated using a 0 to 3+ scale (0, completely negative, 1+ faint membranous positivity, 2+ moderate membranous positivity, 3+ strong circumferential membranous positivity). Cytoplasmic positivity was noted but not incorporated into the final scoring. Score of 3+ membranous staining was considered as high expression.

Differential PCR. Ten-micron sections of formalin-fixed, paraffin-embedded tissues from each case were placed in eppendorf tube. The paraffin was removed from the sections using xylene and alcohol. The sections were rehydrated with lysis buffer (pH=8.5) 15 hours at 55 ˚C with addition of proteinase K. After digestion with proteinase K, phenol:chloroform extractions were applied. DNA was precipitated with 100% alcohol and resuspended in nuclease-free water. Five μl of nucleic acid was added to 25 μl PCR master mix (Promega, USA). Each reaction tube contained the primers for target gene (c-erbB-2) and control gene (Dopamin). The primers used were as follows [8]:

For c-erbB-2

(2122-2141)5' CCTCTGACGTCCATCATCCT 3' (sense primer)

(2217-2196) 5' ATCTTCTGCTGCCGTCCGTCGCTT 3'(antisense primer)

Dopamin receptor

(6-27) 5' CCACTGAATCTGTCCTGGTATG 3' (sense primer)

(118-96) 5'GTGTGGCATAGTAGTTGTAGTGG 3' (antisense primer).

Amplification was performed by 35 PCR cycles on thermal cycler, consisting of three 1 minute steps at 95 ˚C, 55 ˚C and 72 ˚C. After amplification the final product was then fractionated on vertical 8% polyacrylamide gel, stained with ethidium bromide and photographed. The intensity of the c-erbB-2 band and dopamin band was determined for each specimen by means of "NIH Image 1.60" densitometer system. The results were expressed as the ratio: intensity of the c-erbB-2 band/intensity of dopamin band.

Evaluation of c-erbB-2 by differential PCR. The differential PCR assay results were categorized as: c-erbB-2/Dopamin receptor<1 single copy, 1,2≤c-erbB-2/Dopamin receptor <2 low level amplification and ≥2 as high level amplification.

Statistical methods. Statistical significance was set at $p<0.05$. Data were analyzed by the Mann-Whitney U test and x^2 test.

Results

The mean age of the patients was 50 years, ranging from 25 to 86 years. As regards the histologic type, 30 infiltrating ductal carcinomas and 30 infiltrating lobular carcinomas were considered. Fifteen cases of each group were node-positive and the rest was node-negative.

IHC for c-erbB-2. Overexpression of c-erbB-2 oncoprotein: 15 of the 60 cases had high expression (+3), 12 of them had low expression and 33 of them were negative (Fig. 1, Tab. 1).

Differential PCR for c-erbB-2. Gene amplification was detected in 41.6% of the cases. Ten of them had high level, 15 of them had low level and 35 of 60 cases were single copy (negative). Control cases had single copy (Fig. 2, Tab. 1).

Comparison DPCR and IHC for c-erbB-2. Two of the 12 IHC 2+ cases were found by PCR as single copy (negative) and 5 of the 15 IHC 3+ cases were detected as low level of amplification. Finally, there was a high correlation between the results of protein expression and gene amplification ($p=0.000$) (Tab. 1).

c-erbB-2 amplification/overexpression and the other prognostic parameters. Relationship between prognostic indicators of breast cancer and c-erbB-2 protein expression and c-erbB-2 gene amplification are shown in Table 2. Amplification and expression were significantly associated with tumor grade, number of the involved axillary lymph nodes and presence of lymphatic invasion ($p<0.001$). Age, tumor size, histologic type, presence of positive lymph nodes, es-

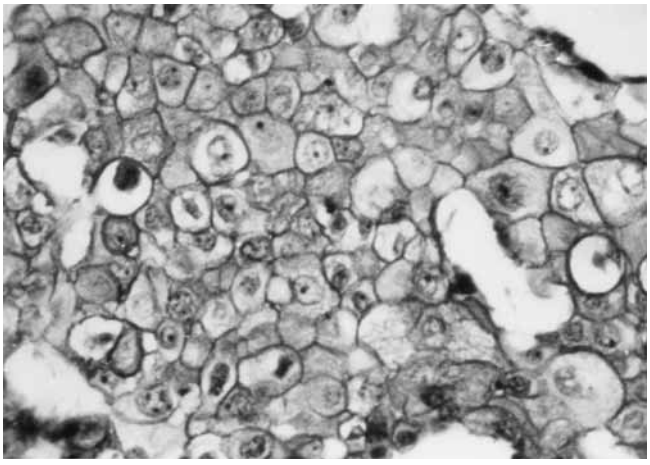

Figure 1. Immunohistochemical assay of c-erbB-2 protein expression. Infiltrating ductal carcinoma shows strong (3+) membrane staining (IHC X 400).

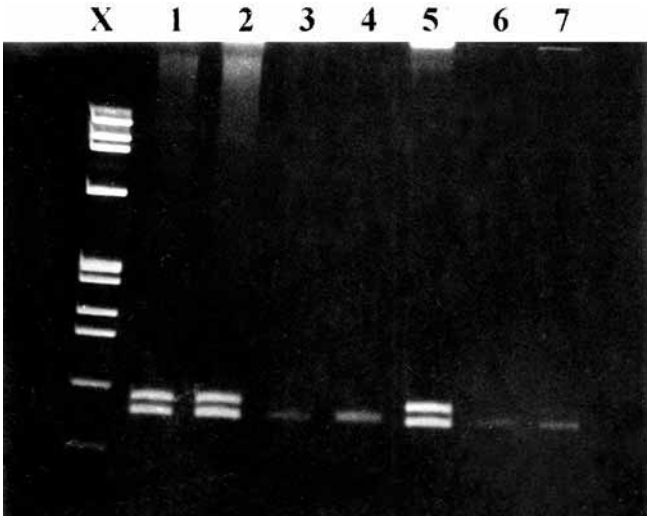

Figure 2. Differential PCR assay of c-erbB-2 gene amplification. Sizes of upper (dopamine receptor) and lower (c-erbB-2) products are 112 and 98 bp, respectively. Samples 1 and 2 have unamplified c-erbB-2 genes (c-erbB-2/dopamine=1). Samples 3, 6 have low level c-erbB-2 amplification (1.2<c-erbB-2/dopamine<2). Samples 4, 7 show high level c-erbB-2 amplification (c-erbB-2/dopamine>2). Sample 5 is a fibroadenoma without c-erbB-2 amplification (c-erbB-2/dopamine<1). X is a molecular size marker.

Table 1. Comparison of c-erbB-2 gene amplification and protein expression

c-erbB-2 expression with IHC	CerbB-2 amplification with differential PCR			
	Single copy (negative)	Low level	High level	Total
Negative	33	–	–	33
Low	2	10	–	12
High	–	5	10	15
Total	35	15	10	60

Chi-square = 77.14; p = 0.000; Kappa = 0.80

Table 2. Correlation of c-erbB-2 protein expression and gene amplification with the conventional prognostic factors in breast carcinoma

	C-erbB-2 expression				p	C-erbB-2 amplification				p
	Negative		Positive			Negative		Positive		
	n	%	n	%		n	%	n	%	
Age										
<50	19	(65.5)	10	(34.5)	0.1	19	(65.5)	10	(34.5)	0.3
≥50	14	(45.2)	17	(54.8)		16	(51.6)	15	(48.4)	
Histologic type										
ductal	15	(50.0)	15	(50.0)	0.6	16	(53.3)	14	(46.7)	0.6
lobular	18	(60.0)	12	(40.0)		19	(63.3)	11	(36.7)	
Tumor size										
<3	12	(70.6	5	(29.4)	0.1	13	(76.5)	4	(23.5)	0.08
≥3	21	(48.8)	22	(51.2)		22	(51.2)	21	(48.8)	
Tumor grade										
I	12	(100)	–	–	0.000	12	(100)	–	–	0.000
II	20	(62.5)	12	(37.5)		22	(68.8)	10	(31.3)	
III	1	(6.3)	15	(93.8)		1	(6.3)	15	(93.8)	
Lymphatic invasion										
positive	8	(33.3)	16	(66.7)	0.006	8	(33.3)	16	(66.7)	0.003
negative	25	(69.4)	11	(30.6)		27	(75.0)	9	(25.0)	
Presence of metastatic lymph node										
positive	13	(43.3)	17	(56.7)	0.1	14	(46.7)	16	(53.3)	0.1
negative	20	(66.7)	10	(33.3)		21	(70.0)	9	(30.0)	
Number of the involved nodes										
1–3	11	(84.6)	2	(15.4)	0.000	12	(78.6)	3	(21.4)	0.003
4↑	2	(11.8)	15	(88.2)		2	(18.8)	13	(81.3)	
Estrogen receptor										
positive	17	(50.0)	17	(50.0)	0.4	18	(52.9)	16	(47.1)	0.4
negative	16	(61.5)	10	(38.5)	17		(65.4)	9	(34.6)	

trogen receptor status were not associated with either expression or gene amplification.

Discussion

Recent studies have showed that c-erbB-2 plays an important prognostic role in the breast carcinomas. The human homologue of the rodent neu gene, referred to as HER-2 or c-erbB-2 is activated by gene amplification that results in overexpression of p185 [1, 3, 10, 15, 16, 19, 22, 24, 27, 28]. This protein can be detected by IHC as a strong membranous staining. A correlation between gene amplification and protein overexpression has been showed in previous studies [9, 13, 26]. It is now clear that c-erbB-2 not only enhances the metastatic potential of breast cancer cells but also associated with drug resistance or sensitivity to specific chemotherapy and hormonal therapy regimens. Also monoclonal antibody directed against the extracellular domain of the c-erbB-2 protein is being used to treat breast carcinomas with overexpression of this protein [5, 19, 27, 29]. These developments necessitate the most reliable assay for evaluating tumors for c-erbB-2. Beside the methods to detect gene amplification (FISH, Western blot), differential

PCR is a semi-quantitative analyzing system that does not require radioactively labeled probes, fresh tissue and can readily be used in formaline fixed-paraffin embedding tissue [8, 20].

In this study, DNA amplification was detected in 25 of 60 (41.6%) of the cases by using differential PCR. There was a high correlation between the amplification and overexpression. But 5 of the 3+ cases with IHC had low level of DNA amplification. However, in approximately 10% of breast lesions, the tumor cells had increased surface receptor expression without gene amplification. This apparently involves some types of transcriptional or posttranscriptional regulation [6]. Insufficient antigen retrieval might be the cause for the false positive staining with IHC. Nineteen of the 60 cases had cytoplasmic positivity without membranous staining and it was not correlated with gene amplification (p=0.4). This result supports the idea that cytoplasmic background staining should not be construed as a positive reaction and that only a distinct membrane staining reaction can be interpreted as positive.

IHC has been the predominant method used by pathologists, however it can be easily affected by the temperature of the paraffin embedding procedure, nature of tissue fixation, prolonged storage, all of which can influence c-erbB-2 protein antigen-loss [8, 10, 19, 26]. In this series, gene amplification was achieved in 10 of 12 2+ IHC cases. This finding suggests that a combination of IHC and differential PCR analyses should be performed especially in all 2+ IHC cases to optimize c-erbB-2 testing.

Much of the previously published data concern the association between c-erbB-2 expression and conventional prognostic factors [1–3, 5, 7–10, 13, 15, 16, 19, 20, 22, 24, 26–29]. The results of these studies have varied but several studies have found consistent results. Firstly, Slamon et al [22] showed a positive association between c-erbB-2 expression and the number of involved axillary nodes. Rosen et al [17], Bianchi et al [2] suggested that c-erbB-2 is not a reliable prognostic indicator for patients with axillary lymph node negative carcinomas, but this subject still remains controversial.

Some studies have postulated the reverse interaction with c-erbB-2/ER and the resistance to antiestrogen therapy in the case of c-erbB-2 overexpression [1, 4, 12, 14, 21]. Saceda et al have demonstrated that the activation of erb receptor inactivated the ER by the heregulin and gp-30 ligands [21]. On the other hand, there are some studies which showed that these two parameters are independent factors but the patients with c-erbB-2+/ER- had worse prognosis [11, 23]. Also there are limited studies about the comparative incidence and significance of c-erbB-2 amplification for lobular and ductal breast carcinomas. Recently, Rosenthal et al [18] detected the gene amplification at a significantly lower rate in lobular breast carcinoma compared with ductal breast carcinoma and when present in lobular type c-erbB-2 gene amplification is a significant prognostic factor. But Rilke et al [16], Uehara et al [25], could not show any correlation with the histologic type.

Contradictory results have been reported about tumor size and c-erbB-2 amplification [1, 11, 12, 14, 23, 30]. Rilke et al [16] indicated that this oncogene product is more frequently over-expressed in large tumors than in small ones.

In this study, we found statistically significant association between c-erbB-2 amplification, overexpression and tumor grade, lymphatic invasion. Although there was no association between the presence of positive axillary lymph node, it was significantly associated with the number of involved lymph nodes. The correlation between the number of involved lymph nodes and the presence of lymphatic invasion and c-erbB-2 put out an association with this oncogene and enhanced malignancy of tumor cells. There was no significant association between c-erbB-2 and age, tumor size, histologic type and estrogen receptor status.

In conclusion, differential PCR is an accurate, economic, semi-quantitative method for assessing c-erbB-2 gene amplification. It can be performed on paraffin-embedded tissue. Although IHC assay as a routine diagnostic technique may be found more suitable than PCR, a combination of IHC and differential PCR may be of value for 2+ IHC cases or for cases whose protein products are not highly expressed and detectable with antibodies. We found out that c-erbB-2 is a factor independent from age, tumor size, histologic type and estrogen receptor status but associated with tumor grade, number of involved lymph nodes and lymphatic invasion. Our results indicate that c-erbB-2 expression/amplification is more frequent in tumors of advanced stage and in tumors that are more poorly differentiated. Additional studies aimed to find the best method for detecting c-erbB-2 and its relation with the other parameters will likely provide valuable insights into the prognosis and treatment of breast carcinoma.

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