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Comparison of Immunohistochemistry and Fluorescence In Situ Hybridization for the Analysis of HER2/neu and Topoisomerase II-alpha Status in Human Breast Cancer

İnsan Meme Kanserinde Topoizomeraz II-alfa ve HER2/neu Saptanmasında İmmünohistokimya ve Floresan İn Situ Hibridizasyon Yöntemlerinin Karşılaştırılması

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ABSTRACT

Objective: HER2/neu is overexpressed/amplified in 20% of breast cancers. HER2/neu status plays a role in determining the patients who might benefit from hormonal therapy and targeted therapy with Trastuzumab. The main cause of HER2/neu overexpression is gene amplification. 10-25% of patients show Topoisomerase II-alpha gene alterations with HER2/neu amplification. The objective of this study was to compare and standardize immunohistochemical and fluorescence in situ hybridization methods for the analysis of HER2/neu and Topoisomerase II-alpha.

Material and Method: 78 cases with invasive breast cancer were selected from the archives. Anti-human HER2/neu, and Topoisomerase II-alpha antibodies were used to determine protein expression levels by immunohistochemistry; TOP2A/HER2/CEP17 probe set was used to examine genomic alterations by fluorescence in situ hybridization.

Result: HER2/neu overexpression was observed in 59% and HER2/neu amplification in 44.9% of the cases. The mean percentage of tumor cells that expressed Topoisomerase II-alpha was 25.9%. 12 cases with Topoisomerase II-alpha amplification (15.4%) also amplified with HER2/neu. The association between HER2/neu and Topoisomerase II-alpha amplification and their expression levels was statistically significant ($p<0.01$, $p=0.005$). The concordance between immunohistochemistry and fluorescence in situ hybridization was 71.7% in 3+ and 11.7% in 2+ cases. Two patients showed chromosome 17 polysomy.

Conclusion: The concordance between immunohistochemistry and fluorescence in situ hybridization was low in 3+ and 2+ cases. Immunohistochemistry and fluorescence in situ hybridization should be performed together until the standardization of the whole process that affects immunohistochemistry and fluorescence in situ hybridization results. If Topoisomerase II-alpha gene alterations are proven by clinical studies to affect the tumor response to the anthracyclines, it will be appropriate to detect these alterations by fluorescence in situ hybridization.

Key Words: Breast cancer, Genes, HER2/neu, DNA topoisomerase II-alpha, Immunohistochemistry, Fluorescence in situ hybridization

ÖZ

Amaç: HER2/neu aşırı ekspresyonu/amplifikasyonu meme kanserli olguların yaklaşık %20'sinde görülmektedir. HER2/neu durumu, hormonal ajanlar ve Trastuzumab tedavisine yanıt konusunda belirleyicidir. HER2/neu aşırı ekspresyonunun en önemli sebebi gen amplifikasyonudur. HER2/neu amplifiye tümörlerde %10-25 oranında Topoizomeraz II-alfa geninde değişiklikler görülmektedir. Bu çalışmada HER2/neu ve Topoizomeraz II-alfa'nın saptanmasında, immünohistokimya ve floresan in situ hibridizasyon yöntemlerinin karşılaştırılması ve standardizasyonu amaçlanmıştır.

Gereç ve Yöntem: Arşivden meme karsinomu tanısı almış 78 olgu seçildi. Anti-insan HER2/neu ve Topoizomeraz II-alfa antikorları kullanılarak HER2/neu ve Topoizomeraz II-alfa protein düzeyleri immünohistokimyasal yöntem ile; gen değişiklikleri TOP2A/HER2/CEP17 probu kullanılarak floresan in situ hibridizasyon yöntemi ile araştırıldı.

Bulgular: Olguların %59'unda HER2/neu aşırı ekspresyonu, %44,9'unda HER2/neu amplifikasyonu saptandı. Topoizomeraz II-alfa eksprese eden tümör hücrelerinin ortalaması %25,9 olarak bulundu. Topoizomeraz II-alfa amplifikasyonu olan 12 olgu (%15,4) aynı zamanda HER2/neu amplifiye idi. HER2/neu ve Topoizomeraz II-alfa amplifikasyonu ile kendi ekspresyon düzeyleri arasındaki ilişki istatistiksel olarak anlamlı olarak tespit edildi (sırasıyla $p<0,01$, $p=0,005$). Floresan in situ hibridizasyon ile immünohistokimya arasındaki uyum 3(+) olgularda %71,7, 2(+)'lerde ise %11,7 olarak bulundu. İki olguda kromozom 17 polizomisi saptandı.

Sonuç: HER2/neu durumunu belirlemede 3(+) ve 2(+) olgularda, immünohistokimyasal ve floresan in situ hibridizasyon arasındaki uyum düşüktür. Sonuçları etkileyen tüm süreçler standardize edilene kadar, bu yöntemler birlikte uygulanmalıdır. Antrasiklinlere karşı tümör cevabını etkilediği klinik çalışmalarla kanıtlanırsa, Topoizomeraz II-alfa gen değişikliklerinin floresan in situ hibridizasyon yöntemi ile araştırılması uygundur.

Anahtar Sözcükler: Meme kanseri, Genler, HER2/neu, DNA topoizomeraz II alfa, İmmünohistokimya, Floresan in situ hibridizasyon

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INTRODUCTION

HER2/neu, ErbB2 the human epidermal growth factor receptor 2 gene, has prognostic importance in breast cancer and codes the transmembrane cell surface receptor protein possessing intrinsic tyrosine kinase activity (1). The HER2/neu protein is active in cell proliferation just like the epidermal growth factor receptor (EGFR) (2). HER2/neu overexpression is seen in 20-30% of breast cancer cases, due to gene amplification 95% of the time (3,4). Slamon et al. have concluded that HER2/neu amplification decreased general survival and disease-free survival in cases with lymph node metastases (5). Determining HER2/neu status is important for choosing cases suitable for treatment with the recombinant human anti-HER2/neu antibody Trastuzumab (Herceptin®, Genenctech, CA, USA) used for targeted treatment.

HER2/neu status may be determined with fluorescent in situ hybridization (FISH), chromogenic in situ hybridization (CISH), silver-enhanced in situ hybridization (SISH), polymerase chain reaction (PCR) and Southern Blot at the DNA level; with Northern Blot and reverse transcription PCR (RT-PCR) at the mRNA level; and immunohistochemistry (IHC) and Western Blot at the protein level.

The method to be chosen for routine work should be applicable to archived paraffin blocks. Morphological detail evaluation of the tissue should be easy and quick.

FISH is the current gold standard in detecting HER2/neu amplification. Its disadvantages are the expense, long technical procedures, the need for a fluorescent microscope and the difficulty in detecting the area with tumor under the fluorescent light.

Although cases where overexpression has been found with IHC but no amplification with FISH make up 3-8% of some series, there are also series with figures of 29-31% (6). FISH and IHC cases are largely consistent in 0, 1(+), 3(+) cases but amplification is found by FISH in only 40% of 2(+) cases.

The HER2/neu gene region and chromosome 17 centromere region copy numbers increase in the presence of chromosome 17 polysomy. Chromosome polysomy is thought to cause HER2/neu protein overexpression but no increase in HER2/neu mRNA expression has been found with the in situ hybridization (ISH) method in polysomic cases (5, 7-9).

Changes (amplification, deletion) may also be seen at the Topoisomerase 2 alpha (TOP2A) gene region close to the HER2/neu locus on chromosome 17 in breast cancer cases (10). The protein coded by the TOP2A gene helps regional unwinding of the DNA double helix during replication and transcription in the cells.

Anthracycline-group agents used in the adjuvant chemotherapy of breast cancer cases act by inhibiting the TOP2A enzyme (11,12). Our aim in this study was to determine by the IHC and FISH methods HER2/neu shown to have both a prognostic and predictive role in breast cancer and TOP2A thought to have a role in deciding whether to use an adjuvant chemotherapy regime, and to standardize these methods.

MATERIAL and METHOD

A total of 78 cases diagnosed as breast cancer at the Ege University Medical Faculty Pathology Department between 2005 and 2007 were included in our study. The IHC method was used for HER2/neu and TOP2A protein levels while changes in HER2/neu, TOP2A and the chromosome 17 centromere region (CEP17) localized on chromosome 17 were investigated with the FISH method in all cases.

Two sections were obtained from the formalin-fixated, paraffin-embedded tumor blocks to test HER2/neu and TOP2A with the IHC method and one section to search for the TOP2A/HER2/CEP17 gene regions with the FISH method for a total of three serial sections, 5 μm thick, placed on a positive charged slide and kept overnight in the 56°C autoclave.

Fluorescent in situ hybridization method and Evaluation

The FDA-approved PathVysion®, TOP2A/HER2/neu/CEP17 tricolor probe was used (Abbott/Vysis, IL, USA). It contains the direct Spectrum Green fluorochrome-labeled DNA probe specific to the 17q11.2-q12 region (HER2/neu), the direct Spectrum Orange fluorochrome-labeled DNA probe specific to the 17q21-q22 region (TOP2A) and the direct Spectrum Aqua fluorochrome-labeled DNA probe specific to the 17p11.1-q11.1 centromeric region (CEP17/alpha satellite).

The preparations were deparaffinized for 45 minutes in a water bath in Skip Devax working solution at an internal temperature of 80°C and were kept in an enzyme solution in the 37°C autoclave for 1 hour and 45 minutes. They were then kept in a 73°C formamide and saline-sodium citrate buffer (SSC) solution for 5 minutes for denaturation and hybridization was achieved in a hybridizer device overnight. Posthybridization washing was performed and the samples kept at -20°C for 20 minutes after 10 μl of 4,6-diaminido-2-phenylindole dihydrochloride (DAPI) was added as a drop.

The Zeiss Axioscope II FS (Carl Zeiss, Jena, Germany) fluorescent microscope was used and each field of the section was analyzed with the x100 objective.

Following hybridization, the red signals indicated TOP2A, green signals HER2/neu, and the aqua signals the CEP 17 gene region. All other DNA regions were seen as blue with DAPI. Two signals from the CEP17 gene region were used as internal control.

The criteria of the "American Society of Clinical Oncology / College of American Pathologists" (ASCO/CAP) protocol published in 2007 were used when evaluating HER2/neu gene amplification with FISH (13):

- Negative: HER2/neu gene copy number < 4 /nucleus, HER2/neu/CEP 17 <1.8
- Equivocal (suspect positive): HER2/neu gene copy number = 4-6, HER2/neu/CEP 17 = 1.8 - 2.2
- Positive: HER2/neu gene copy number >6 or HER2/neu/CEP17 >2.2 in 20 tumor cells in two different fields

The criterion used to determine TOP2A amplification was TOP2A/CEP17 >2.

The criterion used to determine chromosome 17 polysomy was CEP17 >3.

Immunohistochemical method and Evaluation

Mouse Topoisomerase 2 alpha monoclonal antibody (clone KiS1, dilution 1/100, Dako, Denmark); ready-to-use peroxidase kit (Ultravision LP Value Detection System, Large Volume HRP Polymer, Labvision, CA, USA) and DAB Chromogene (Thermo Scientific DAB Substrate System, Labvision, CA, USA) were used with manual staining for Topoisomerase 2 alpha. The positive control was a tonsillar tissue section

The FDA-approved Polyclonal Rabbit Anti-human HER2/neu antibody (clone AO485, dilution 1/300, Dako, Denmark) and non-biotinylated HRP multimer based, hydrogen peroxide substrate and 3, 3' – diaminobenzidine tetrahydrochloride (DAB) chromogen-containing staining kit (ultraView™ Universal DAB Detection Kit, Catalog number 760-500, Ventana, AZ, USA) and a fully automated immunohistochemistry and in situ hybridization staining system (Ventana BenchMark XT, AZ, USA) were used for HER2/neu.

The HercepTest IHC kit evaluation table (0; no staining, 1+; weak interrupted membranous staining, 2+; weak-moderate uninterrupted membranous staining (>10%), 3+; strong uninterrupted membranous staining (>10%)) was used when evaluating HER2/neu overexpression. 0 and 1+ were accepted as negative, while 2+ was suspect positive and 3+ was positive. There are no thresholds in the literature for TOP2A overexpression and we therefore scored our cases according to the percentage of tumor cells with strong nuclear positivity and statistical evaluations were used to investigate whether there was a relation between these expression percentages and amplification.

Statistical Evaluation

Statistical evaluation was performed with the SPSS (version 15.0) software. The results were expressed as mean value ± standard error. The relationship between HER2/neu overexpression and TOP2A amplification, ER, PR, and histological grade was evaluated with the "chi-square test" and that between TOP2A expression with the "Kruskal Wallis" test. Data distribution was analyzed with the Kolmogorov-Smirnov test. The relationship between TOP2A amplification and expression with age and diameter was analyzed with "Student's t-test" and the relationship with the expression percentages of TOP2A and HER2/neu with the "Mann –Whitney U test". A p value less than 0.05 was considered statistically significant for all analyses.

RESULTS

The 78 cases were distributed as follows: 48 (61.5%) invasive ductal carcinomas (IDC), 5 (16.4%) pleomorphic lobular carcinomas (PLC), 5 (6.4%) apocrine carcinomas, 4 (5.1%) invasive lobular carcinomas (ILC), 2 (2.6%) glycogen-rich breast carcinomas and 2 (2.6%) medullary carcinomas. The remaining 12 cases were one each (1.3%) of invasive micropapillary carcinoma (IMC), IDC+IMC, IDC with prominent intraductal component, colloid carcinoma, IDC+colloid carcinoma, IDC+apocrine carcinoma, IDC+glycogen-rich breast carcinoma, IDC+signet ring cell carcinoma, histiocytoid carcinoma, invasive papillary carcinoma, IDC+ILC, and IDC+PLC. The diagnoses were classified into five subgroup as IDC, ILC, PLC, mixed carcinomas containing an IDC component, and others so that subgroups with only a few cases could be combined.

The mean age of the cases was 55.3±1.48. The mean tumor size was 2.3±0.12 cm. HER2/neu expression was as follows: 0 in 17.9%, 1(+) in 1.3%, 2(+) in 21.8%, and 3(+) in 59%. The mean percentage of TOP2A-expressing tumor cells was 25.93±2.12%. We found no statistically significant difference between age, tumor size and HER2/neu overexpression and TOP2A expression level with the histopathological subtypes investigated in five groups (p=0.833, p=0.35, p=0.513, p=0.873, p=0.757, p=0.717 respectively). HER2/neu overexpression was found to show a statistically significant relation with high histological grade and higher ratios of metastatic lymph node number/dissected total lymph node number (p=0.002, p=0.001 respectively). There was a statistically significant relationship between HER2/neu overexpression and ER and PR negativity (p=0.007, p=0.005 respectively)..

We found HER2/neu amplification in 35 (44.9%) and TOP2A in 12 (15.4%) of the 79 cases. There was also concurrent HER2/neu amplification (co-amplification) in 12 (100%) cases. There was a statistically significant relationship between TOP2A and HER2/neu amplification (p<0.01). There was no significant relationship between TOP2A amplification and ER and PR status, age, tumor size and the histological subtypes (p=0.392, p=0.884, p=0.752, p=0.452, p=0.674 respectively) while there was a statistically significant relationship with histological grade (p=0.046).

We found a statistically significant relation between HER2/neu overexpression and HER2/neu amplification (p<0.01) (Figure 1A-C). Of the amplified cases, 71.7% was HER2/neu 3(+). This means that the consistency between IHC and FISH in 3(+) cases was 71.7%. We found amplification in 2 (11.7) of the 17 IHC 2(+) cases. There was no amplification with FISH in IHC 1(+) and 0 cases. The sensitivity and specificity as regards determination of amplification when HER2/neu expression was about 55% were calculated as 95% and 80% respectively (Figure 2A-C). The FISH and IHC images of HER2/neu 3+ cases are presented in Figure 1A-C and Figure 2A-C. Chromosome 17 polysomy was found in two cases that were IHC 3(+) but showed no amplification.

We found a statistically significant relationship between TOP2A amplification and expression levels (p=0.005). However, we could not determine a reliable expression threshold value that could be used to specify TOP2A amplification. The sensitivity was 83% and the specificity

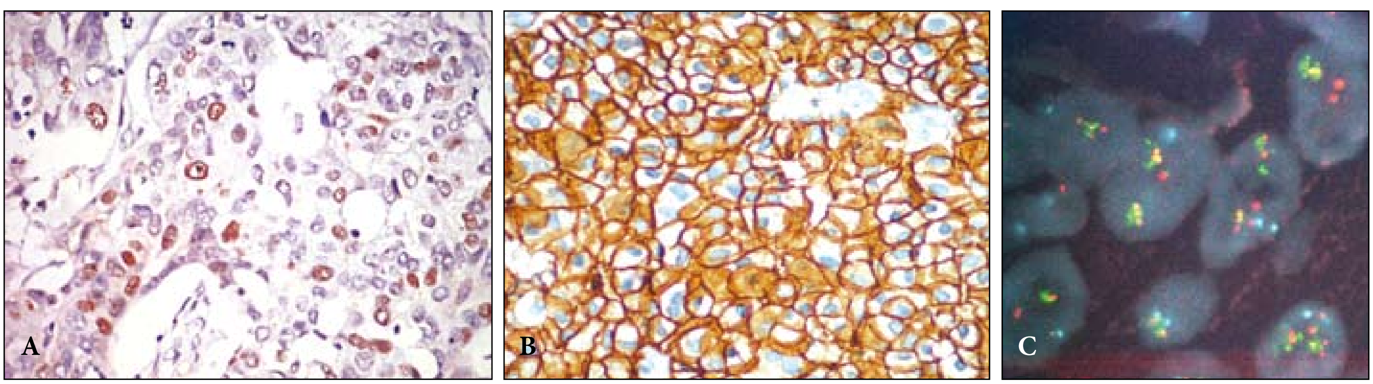

Figure 1: Invasive ductal carcinoma. **(A)** TOP2A expression in 20% of tumor cells, **(B)** HER2/neu 3(+) tumor cells (A,B IHC, DAB, x400), **(C)** An increase in the number of red and green signals in tumor cells indicating the co-amplification of TOP2A and HER2/neu (FISH, DAPI).

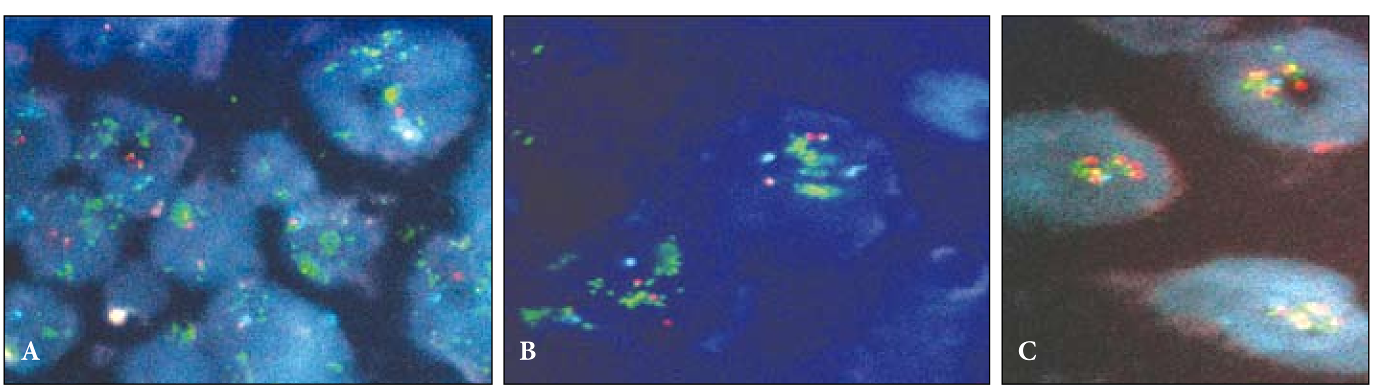

Figure 2: (A) Increase in the number of green signals in tumor cells indicating HER2/neu amplification, **(B)** Increase in number and small clusters of green signals related to the HER2/neu gene region in pleomorphic lobular carcinoma cells showing HER2/neu 3+ and amplified pleomorphic lobular carcinoma case showing small clusters and increase in number of green signals, **(C)** HER2/neu and TOP2A co-amplification; Tumor cells showing an increase in number of the green signals related to the HER2/neu gene region and the red signals related to the TOP2A gene region (FISH, DAPI).

43% when the expression level was 22.5%. No TOP2A deletion was observed in the cases.

DISCUSSION

HER2/neu has an important role in the oncogenesis of 20-30% of breast cancer cases (3,5). The reasons for inconsistencies between HER2/neu gene and protein product can be chromosome 17 polysomy, overexpression at the transcriptional or posttranslational steps, low sensitivity and specificity of the primary antibody used in IHC, problems with tissue fixation and processing and aggressive antigen exposure methods (14,15).

The most common and least expensive method used to determine HER2/neu at the protein level is currently immunohistochemistry. The most important advantages are that it is easy and can be quickly performed, is repeatable and can be evaluated with the light microscope. However, it is affected by the fixation duration, fixative type, tissue processing method, antigen exposure method, primary antibody sensitivity/specificity and dilution rates.

For optimum results, the material should be put in buffered 10% formalin within one hour at the latest and should stay in the fixating solution for 6-48 hours.

We also suffered intensively from problems related to the fixation. Autolysis was seen in the tissues at times due to problems with getting surgical material to our department and late fixation.

The correlation between IHC 3(+) cases and FISH varies between 70% and 100% in reports and is generally about 90% (16,17). The rate in our case was 71.7%.

The relatively low correlation between HER2/neu overexpression and amplification in our study compared to the literature is probably due to the problems with fixation that affect IHC. We also found that consulted cases where the material fixation and processing had been performed at an outside center had a negative effect on our IHC results.

Only homogenous dark membranous staining should be taken into account when evaluating the HercepTest IHC kit. IHC should be repeated if cytoplasmic staining may have suppressed membranous staining. In situ areas should not be taken into account while scoring

Cases that were IHC 2(+) and 3(+) were accepted to be positive for overexpression. However, there is little relationship between 2(+) status and gene amplification and these cases were then interpreted as weak positive or suspect positive. ASCO/CAP published guidelines in 2007 for the best interpretation of HER2/neu (13). The threshold value for IHC 3(+) was increased from 10% to 30%. This made some cases previously evaluated as 3(+) transfer to the 2(+) category.

Amplification is found at a rate of 6-62% with FISH in IHC 2(+) cases (18-21). This rate is 25-30% in general (15,16,22,23). The rate in our study was 11.8%. FISH must be used before trastuzumab treatment in 2(+) cases due to these varying changes in the literature.

CAP states that it is better to provide a percentage as there is a direct relation between overexpression levels and the presence of amplification (24). Both the staining intensity and the percentage of stained cells were evaluated when looking for HER2/neu overexpression in our study and there was a significant relation between the increase in HER2/neu expression percentages and amplification ($p<0.01$). The sensitivity was 95% and specificity 80% at an expression level of 55%.

Polyclonal and monoclonal antibodies are used to determine HER2/neu. Lebeau et al. compared the results of five monoclonal (9G6, 3B5, CB11, TAB250, GSF-HER2/neu) and two polyclonal antibodies (A8010, AO485) for determining HER2/neu using the IHC method with the results of the Hercep Test. Overexpression was found at a rate of 26-27% with monoclonal antibodies and 42% with the AO485 polyclonal antibody and HercepTest. Amplification was found in 28% of these cases with FISH. Amplification was not found in 85% of the cases that were 2(+) with the HercepTest. They observed that polyclonal antibodies were superior to monoclonal ones in determining low levels of gene amplifications but also had a high false positivity rate (16).

Benign breast epithelium must be evaluated carefully especially when a polyclonal antibody is being used. IHC should be repeated for normal epithelial staining that exceeds the 1(+) criteria.

The gold standard method to determine the HER2/neu gene region is FISH. The ASCO/CAP recommendations are to limit work to FISH 2(+) cases until there is 90% consistency for 0 and 3+ cases and 95% consistency for 1(+) cases (13).

A single-color HER2/neu DNA probe or two-color HER2/neu/CEP17 probe can be used for the FISH method. The chromosome 17 centromere region can be used as an internal control for two-color probes. It is also more advantageous as it can also show polysomic cases.

We used the three-color TOP2A/HER2/CEP17 probe in our study. We also investigated the incidence of HER2/neu and TOP2A gene changes seen together in this way.

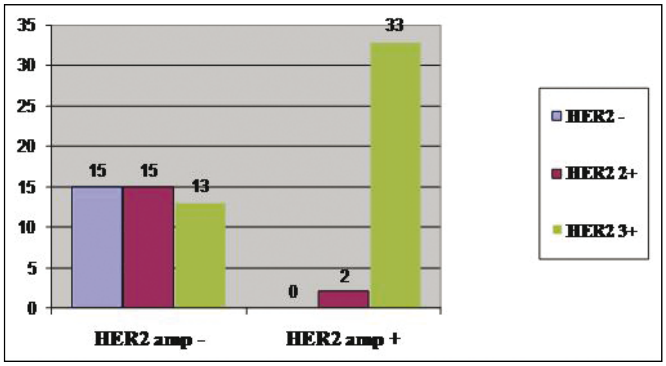

Figure 3: Relationship of HER2/neu amplification with HER2/neu overexpression.

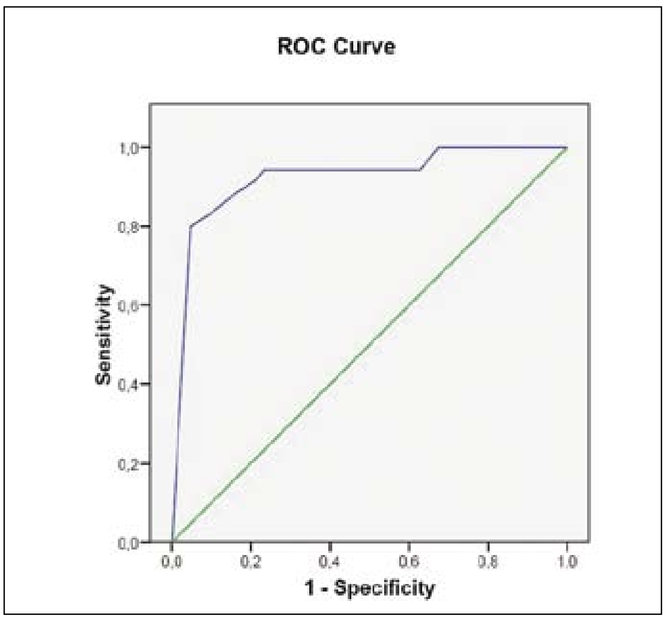

Figure 4: Sensitivity and specificity of HER2/neu expression percentages in determining HER2/neu amplification.

It is more difficult to evaluate the three-color probe than the two-color or single-color probes as the TOP2A and HER2/neu gene regions are located close together on chromosome 17 and the signals can sometimes overlap and suppress each other. Another difficulty is that the aqua-colored signals of the CEP17 region are difficult to discern on the blue background caused by the DAPI that delineates cell borders. This can lead to overlooking polysomies.

A FISH sample suitable for evaluation should have homogenous signals, optimum enzymatic digestion and high nuclear resolution. The procedure should be repeated if there is more than 10% signal at the baseline, the autofluorescence level is high and if unexpected results are obtained from control samples.

We sometimes had problems with the enzymatic digestion and deparaffinization steps in the samples we used FISH on in our study. Some of the problems were caused by the inability to maintain the reaction temperature at which the solutions were active and another could be the simultaneous study of many samples. The paraffin covered the cells and signals like a cloud and made evaluation difficult when full deparaffinization was not achieved.

The above-mentioned factors were effective in addition to the problems encountered with IHC in the 11 cases where HER2/neu was 3(+) without amplification other than polysomic cases.

HER2/neu amplification is seen at a rate of 18-20% in breast carcinoma. We found amplification in 44.9% of our cases. This higher rate than the literature may be due to the fact that 59% of our cases were 3(+).

We included 4 ILC and 4 PLC cases in our study. Three cases were 3+, 3 were 2+, and 2 were (-). Amplification was found in only one PLK case that was HER2/neu 3(+).

TOP2A gene changes are also seen in HER2/neu amplified tumors (25). However, results of recent studies indicate that TOP2A gene changes can also be seen without HER2/neu amplification (26). Analyzing the relationship of TOP2A expression levels with amplification in our study showed that the sensitivity was 80% and the specificity 65% when the 25% value was accepted as the threshold. We therefore felt this was not a reliable threshold value in determining amplification.

Cytotoxic agents including anthracyclines that are TOP2A inhibitors are frequently used as adjuvant treatment of breast cancer. However, anthracyclines can increase the risk of acute leukemia and cardiac damage (27). TOP2A is the direct molecular target of anthracyclines. In vitro studies have shown that sensitivity to TOP2A-inhibiting agents depends on the TOP2A expression levels of the tumor cells (28). Deletion leads to decreased TOP2A levels and the development of resistance against TOP2A inhibitors (29). Top2 amplification has been reported at rates varying from 25 to 40% in breast carcinomas with HER2/neu amplification (10,12,28). We found TOP2A amplification in 15.4% of our cases. These were all HER2/neu-amplified at the same time.

Arriola et al. have found a significant relationship between TOP2A overexpression and MIB1, TOP2A amplification, HER2/neu amplification and overexpression, and ER and PR negativity while none was present between tumor size and lymph node metastasis. They also found a higher percentage of cells expressing TOP2A in TOP2A-amplified cases compared to non-amplified ones (30). We found a significant relationship between TOP2A expression and HER2/neu overexpression in our study. The expression increased especially in cases with a high mitotic index.

Our results are mostly consistent with the literature but we need to increase the consistency with FISH results in 2+ and 3+ cases. This requires ensuring standardization of IHC first. The most important issue is fixating the material with 10% buffered formalin within the hour at the latest.

Paraffin blocks should be sent from external centers to our department when FISH is requested to decrease the problems experienced with the material.

Although there were no cases of false negativity in our study, the false positivity rate was high. Cytoplasmic staining in addition to membranous staining with IHC was also noted at times. This indicates a possible requirement for the use of monoclonal antibodies instead of polyclonal ones.

The tissue fixation and processing should be modified to minimize the technical problems with the FISH method. The deparaffinization should be optimal. Negative and positive control cases should be included in the process for a while. The procedure should not be limited to 2+ cases until the consistency between FISH and IHC reaches the 90% rate. Two-colored probes instead of three-colored ones should be preferred because of evaluation problems.

We believe that the results of our study will help in employing current diagnostic methods and choosing the proper and beneficial treatment models for breast cancer patients.

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