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Evaluating *HER2* Gene Amplification Using Chromogenic In Situ Hybridization (CISH) Method In Comparison To Immunohistochemistry Method in Breast Carcinoma

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Abstract

BACKGROUND: In patients with breast cancer, *HER2* gene expression is of a great importance in reacting to Herceptin treatment. To evaluate this event, immunohistochemistry (IHC) has been done routinely on the basis of scoring it and so the patients were divided into 4 groups. Lately, as there have been disagreements about how to treat score 2 patients, chromogenic in situ hybridization (CISH) and florescence in situ hybridization (FISH) are introduced. Since CISH method is more convenient than FISH for gene amplification study, FISH has been substituted by CISH.

AIM: The current study is conducted in order to investigate whether using CISH is a better method comparison to IHC method for determines HER2 expression in patients with breast cancer in.

METHODS: In this cross-sectional descriptive analytical study, information of 44 female patients with invasive ductal breast cancer were gathered from Imam Reza and Omid Hospital in Mashhad. IHC staining was done for all patients in order to determine the level of HER2 expression, and after scoring them into 4 groups of 0, +1, +2 and +3, CISH staining was carried out for all 4 groups. At the end, results from both methods were statistically evaluated using SPSS software V.22.0.

RESULTS: The average age of patients was 50.2 with the standard deviation of 10.96. Using IHC method was observed that 2.6% (1 patient), 26.3% (10 patients), 65.8% (25 patients) and 5.3% (2 patients) percentage of patients had scores of 0, +1, +2 and +3. On the other hand, CISH method showed 36 patients (90%) with no amplifications and 4 (10%) with sever amplifications. In a comparative study using Fisher's exact test (p = 0.000), we found a significant relation between IHC method and CISH method indicating that all patients showing severe amplifications in CISH method, owned scores of +2 and +3 in IHC method.

CONCLUSION: According to the present study and comparing the results with similar previous studies, it can be concluded that CISH method works highly effective in determining HER2 expression level in patients with breast cancer. This method is also able to determine the status of patients with score +2 in IHC for their treatment with herceptin.

Introduction

Breast cancer is the second death-causing cancer amongst women worldwide and is also the main death cause of women between 20 and 40 [1]. Breast cancer is the most prevalent malignancy in Middle Eastern women [2]. Previous review studies on breast cancer conducted in Iran, haves also shown an occurrence of about 17.2-22 cases in 100,000 individuals [3]. About 72% of patients in Iran had a tumor bigger than 2 centimeters at a time of diagnosis diagnosed [3].

In order to treat breast cancer patients who need to systemic or complementary treatment, after local treatment, there are three remedial processes including hormonal therapy, chemotherapy and human monoclonal antibody therapy like anti

Herceptin. Monoclonal antibody therapy which in this particular cases the human monoclonal antibody would be anti-herceptin. Choosing between different treatment processes depends on how the patient reacts to the hormonal therapy and their level of HER2 expression [4]. HER2 is an oncogene coding a 185-kDa transmembrane glycoprotein expressing intracellular tyrosine kinase activities. HER2 receptor belongs to epidermal growth factor receptors (EGFR) which effects on activation of cascades cellular mechanisms leading to growth and development [5, 6]. Increased gene expression of HER2 is observed in 18 to 20 percent of breast cancer cases [5] [7] [8]. Patients with high levels of HER2 expression respond better to medications like Herceptin. Also, the level of HER2 can expose patient's sensitivity or resistance to chemotropic drugs [9]. Furthermore, recent studies have shown that patients with HER2 expression are resistant to hormonal therapies [10].

Determining HER2 level is a necessity for choosing the best treatment for breast cancer patients and is done through three methods: IHC, FISH, CISH. In IHC method, patients are categorized into 4 scores, which due to mismatched results between different labs and therapy of choice disagreements, it is necessary to recheck HER2 level with either CISH or FISH. Since FISH method also has some drawbacks such as limitations on keeping results, high costs and the need of a florescence microscope, CISH is preferred as it offers a much higher accuracy and sensitivity level [7] [11] [12] [13] [14] [15].

In keeping with previous studies in this area, we decided to compare the evaluating competence of CISH and IHC methods in determining HER2 level in breast cancer patients.

Material and Methods

Female patients with invasive ductal breast cancer whose tissue samples and paraffin blocks were kept in Imam Reza (pbuh) and Omid hospital at Mashhad were included in this sectional descriptive analytical study. Cases with not enough tissue samples for IHC or CISH were excluded from the study, as well as those with in situ carcinoma or other form of breast cancer.

IHC staining was done for all the patients to determine HER2 expression level. New sections were prepared from each block to be stained. Blocks were deparaffinized at first and then was added Citrate buffer in 94°C for 30 min. After that, one overnight incubation with monoclonal antibody (HER2-pY 1248, Dako Denmark A/S) was done at 37°C. Diaminobenzidine was used as chromogen in a standard process of Avidin-Biotin- peroxidase staining in IHC method. After slides preparation, each of them was stained individually to determine the HER2 level.

After that, HER2 staining, results were reported in 4 different groups of 0, +1, +2 and +3. Score 0 (negative result is assigned to those cases showing no detectable stained cells or stained cells are observed in less than 10% of tumor cells). Score +1 is assigned to colorfulness with less than 10% weakly stained tumor cells membrane, score +2 includes cases with weakly to partial stained in more than 10% of tumor cells membrane, and +3 is for cases with more than 30% complete and strongly stained tumor cells membrane (Figure 1) [16].

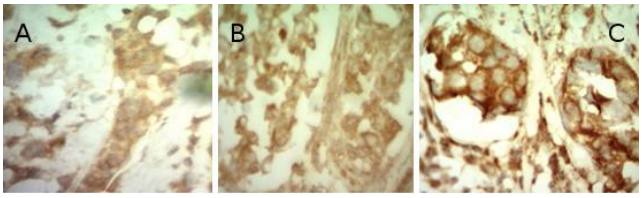

Figure 1: Microscopic pictures of immunohistochemistry samples in order to investigate HER2 gene in breast cancer; a) Score 0-1; b) Score +2; c) Score +3

After scoring the samples using IHC (according to the level of membrane staining intensity and the percentage of stained cells) samples were divided into 4 groups of 0, +1, +2 and +3 and then according to the provided instruction for ZytoDot Kit (ZytoDot 2C, Zytovision, Germany) staining was conducted for all samples. This technique was carried out on 4-5 µm sections of paraffinized tissue blocks.

In site hybridization technique (ISH) refers to an operation which a single-stranded DNA or RNA attach as a probe with a portion of the single-stranded DNA of the cell as a target. In-situ hybridization can be done in a liquid medium or in a solid medium. And the labeled material in probes can be chromogenic enzymes or fluorescent compounds. If hybridization is performed locally in the sample cells, hybridization is called in situ or Hybridization (ISH). So, the CISH method is a hybridization technique in the main site (ISH). In CISH, the generated signal is a kind of color that appears under the Bright field light microscope.

Generally, tissues 4–5 µm thick were mounted on Histogrip-treated microscope slides then dried at 37°C, and baked for 2–4 hours at 60°C. The slides were deparaffinized for 15 min three times in xylene at room temperature (22–27°C) and washed for 2 min three times in 100% ethanol.

These slides were microwaved in SPOT-Light Tissue Heat Pretreatment Buffer for 10 min at 92°C and cleaned for 3 min three times in phosphate-buffered saline (PBS). They were supported with 100 µl SPOT-Light Tissue Pretreatment Enzyme for 10 min at 37°C and washed for 3 min three times in PBS at room temperature. Then, the slides were dehydrated in 70%, 85%, 95%, and 100% ethanol for 2 min, after that air-dried. Probe (15 µl) had been

added to each sample and followed up with a 24 mm × 32 mm coverslip, after that the slides were denatured at 94°C for 3 min and located in a dark humidity box for 16–24 hours at 37°C.After removal of the rubber cement and coverslip, the slides were soaked in 0.5 × SCC buffer in a Coplin jar for 5 min at 75°C. Then they were washed for 2 min three times by PBS-Tween 20 buffer at RT. After Using ZytoDot 2C, Zytovision, Germany CISH kit, the slides were counterstained with 150 µl of Gill-2 hematoxylin and incubated for 3 min. They were then mounted with a coverslip (Figure 2).

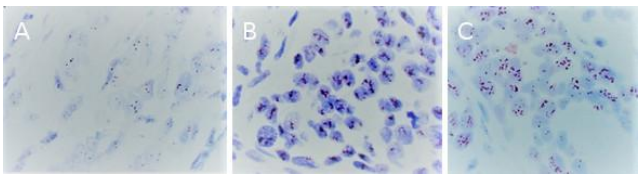

Figure 2: Chromogenic in situ hybridisation (CISH) of HER-2 oncogene in breast cancer; A) With normal HER2 copy number (1000 X) with no amplification; B) CISH on a breast carcinoma with low level HER2 gene amplification (1000 X); C) CISH on a breast carcinoma with high level HER2 gene amplification (1000 X)

Results were observed under the light microscope with 40x and 100x magnifications by 2 experienced pathologists, who were not aware of IHC results beforehand. Invasive tumor areas were recognized and dimensionally analyzed afterwards. HER2 signal, indicating a copy of its gene, was observed as a detectable green spot and signals of chromosome 17 centromere was observed as a red spot, where these two were simply distinguishable. In a normal diploid nucleus without amplifications, 2 green spots and 2 red spots with smooth rounded edges were seen in each nucleus. Then, 100 cells were counted and the ratio of hybridized probe HER2 genes to signals of chromosome 17 centromere was calculated. Necrotic zones, overlapped nuclei and nucleus with undetectable signals were omitted.

Since mitosis can cause extra signals in a few percent of non-neoplastic cells; in cases with heterogeneity, those areas in tumor which showed amplifications, were selected. Samples with the ratio of 2.5 or more were considered as having amplification and those wih the rate less than 2.5 amplifications. Results were categorized based on kit's instructions:

1. Less than 5 copies of HER2 gene in ratio with 2 signals of control (chromosome 17 centromere) in each cell nuclei of 100 counted cells mean there has been no amplifications.

2. Having 5 or more copies of HER2 gene in ratio with 2 signals of control (chromosome 17 centromere), which is sometimes seen as a gene cluster in tumor cell nucleus is considered as amplification.

In cases with no amplifications at diploid cells, 1 to 2 signals and in chromosomal aneuploidy situation, 2 to 4 signals were observed in each nucleus.

As an internal control, lymphocytes and stromal cells were examined and showed 2 signals with smooth rounded edges in each nucleus.

For quality control part, signals must be clear and easily detectable; also it's necessary to have internal control and morphologically healthy cells. Damaged nuclei show extra enzyme digestion which in turn causes dispersed signals or no signals at all.

At the end, statistical analysis was carried out between so called methods using software as below:

Data were analyzed using the statistical analysis software SPSS vs.22 in order to describe the data generally, statistical indexes like, mean, median standard deviation and ranges were used. For the main data analysis Chi-squared test was used to determine the relation between HER2 gene amplification and the score each carcinoma sample gained in IHC. $P < 0.05$ was regarded as statistically significant.

Results

A group of 40 patients with breast cancer were included in this study. The average age of the participant was 50.2 with the standard deviation of 10.96. The youngest patient was 26 and the oldest was 81 years old.

Among all patients, 92.6% were married and 7.4% were single. Tumor grade frequencies for scores I, II and III were 2.7%, 86.5% and 10.8% respectively (NA is assigned to those with no amplification and having amplification in the graph) (Figure 3). Furthermore, based on tumor (T) properties, 10%, 86.7% and 3.3% of patients were classified as T1, T2 and T3 (Figure 4).

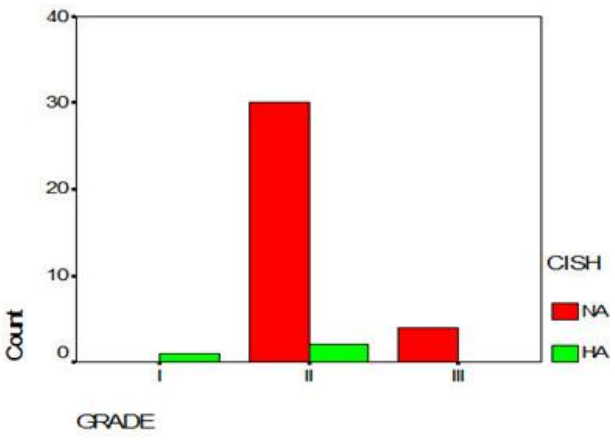

Figure 3: The frequency of grade tumor in samples examined by the method CISH

Evaluations using IHC method showed that respectively 2.6%, 26.3%, 65.8% and 5.3% of patients had scores of 0, +1, +2 and +3. In the other evaluation carried out by CISH method, 36 (90%) samples had not and 4 (10%) had amplification.

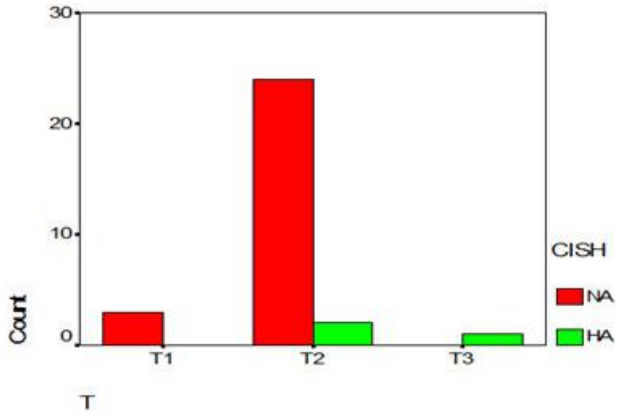

Figure 4: The frequency of tumor characteristics (T) in samples studied by CISH method

In comparative study between IHC and CISH methods, A significant relationship was found between the two methods using Fisher's exact test (p = 0.000) all the samples with no amplification in CISH method had the, scores of +2 and +3 in IHC. It was also recognized that among 25 samples with the score +2 in IHC, only 2 of them showed signs of amplifications in CISH while others were negative for amplification (Figure 5).

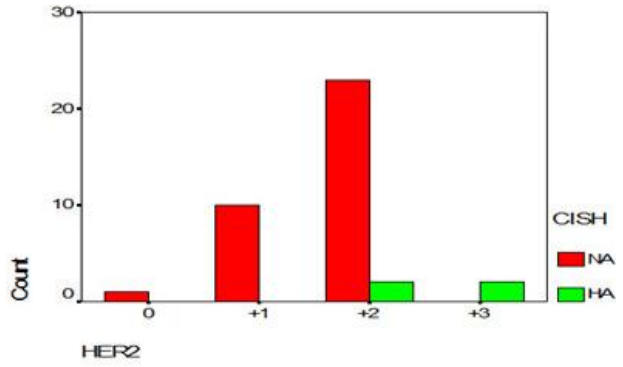

Figure 5: The frequency of results comparing IHC and CISH methods in diagnosis

Also Chi-square test showed a significant relation between CISH and tumor (T) properties (p = 0.009). On the other hand, the T-test didn't show any significant relation between CISH results and patient's age (p = 0.07).

In addition, when comparing between CISH and Tumor Grade, it came to our notice that most of the samples with no amplifications were of level II (p = 0.003). In the other comparison between IHC and tumor (T) properties, Kruskal-Walis test showed no significant relation between IHC and tumor (T) properties (p = 0.991). Also, in comparison between IHC and Tumor Grade, Kruskal-Walis test showed no significant relation between IHC and Tumor Grade (p = 0.993).

Discussion

Breast cancer is the second most prevalent cancer after skin cancer and is the second fatal cancer after lung cancer. It has been established through in various previous studies that HER2 gene amplification has appeared in 18-20% percent of breast cancer patients which in turn increases the mortality rate amongst these patients [17]. Since positive HER2+ patients demonstrate better responses to the treatment with monoclonal Ab Herceptin, it would be advantageous to locate HER2 gene. According to previous studies in determining the status of HER2 IHC method is used first. Soon after, with CISH having such unique properties, this method of staining was chosen to investigate HER2 gene amplification [6] [13] [14] [16] [18] [19] [20] [21].

Therefore, this study was conducted to investigate the quality of diagnosis of CISH and compare CISH with IHC in scoring efficacy of 40 patients with invasive ductal breast cancer. Our results demonstrated a relation between CISH and IHC in scoring patients. This means that all the patients examined in CISH method, also showed high amplifications, manifested high amplification for HER2 gene in IHC method as well (score +2 and score +3). So, for these patients, CISH can be used with high certainty.

In other studies conducted in France on 79 patients with invasive ductal breast cancer and in US involving 161 patient with the same problem confirmed that results obtained from CISH method for determining the level of HER2 gene amplification is more than 90% consistent with others method .and on the other hand, it can be used as a check test for patients with score +2 in IHC method [17] [22]. In a study carried out in Spain addressing the same issue, the compatibility of positive cases in CISH and IHC has been reported to be 72.5% and for negative cases, results of these 2 methods were 100% compatible. However, they could find no significant relationship between CISH, IHC with tumor grade which shows the specificity and novelty of this article. [23]. It has been clarified in other studies that HER2 gene expression level is crucial in how patients would respond to treatment with Herceptin. So in this study, IHC was done routinely and patients were classified into 4 scores and since there have been disagreements regarding treatment of choice for score +2 patients, CISH was used in this study confirmed the efficacy of CISH over IHC and showed a significant relation between CISH an IHC, meaning

that for any sample showing amplification in CISH, there would be a score +2 and +3 in IHC (p = 0.000).

Considering the result from the present study and comparing them with previous research on the issues it can be concluded that since CISH method is a more reliable method, with lower costs and which no need for Epi-fluorescent microscope, and since it also enables the researcher to simultaneously examine morphology and results of hybridization; it can be used as a highly effective method for determining HER2 expression level in patients with breast cancer. However, having some key limitations in this study like small sample size and unavailability of patient's detailed information such as the exact number of involved auxiliary lymph nodes and expanse of metastasis around the hand, it appears that conducting similar using a larger sample size can help in validating the results obtained from this study.

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