Predicting Effects of Clinicopathological Variables on Her2 Gene Amplification by Chromogenic in situ Hybridization (CISH) in IHC Her2 (2+) Breast Cancer Patients: A Study from Iran

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Breast cancer, In situ Hybridization, chromogenic Genes, Her2

ABSTRACT

Background & Objective: The her2 amplification plays an important role in breast cancer management. Therefore, there is a need for using supplementary molecular methods in IHC equivocal cases. Present study has been conducted to determine the effects of clinicopathological variables on her2 gene amplification by chromogenic in situ hybridization (CISH) in IHC Her2 (2+) breast cancer individuals.

Methods: A cross-sectional study was conducted in Zaferanyeh Laboratory collaborated with Shahid Beheshti University of Medical Sciences (Tehran-Iran; 2015-2018). All pathological data related invasive breast cancer patients with equivocal IHC results were included. CISH method was performed as a supplementary technique. The associations between histopathologic variables, status of Ki-67 index, progesterone and estrogen receptors (PR & ER) with her2 amplification by CISH were investigated and analyzed. The level of significance was considered as P-value < 0.05.

Results: Totally, 239 patients with mean age of 53.2 years were studied. CISH identified her2 gene amplification in 51 subjects (21.3%). The type of tumor (invasive ductal carcinoma), the tumor grade, and the value of Ki-67 index were directly correlated with her2 amplification. Significant negative associations were also observed between CISH results and ER and PR expression.

Conclusion: As her2 gene amplification was identified in 21.3% of invasive breast cancer patients with equivocal IHC results, it is supposed that applying CISH method may consider as a potentially valuable supplementary method. Results have also shown that higher grades of tumor, invasive ductal carcinoma, absences of hormone receptors and high Ki-67 index significantly correlated with the her2 amplification.

Original Article

Introduction

The human epidermal growth factor receptor 2 (Her2) with tyrosine kinase activity is a 185-kDa transmembrane glycoprotein. Its gene is located on the long arm of chromosome 17 and expressed at low levels in a variety of body epithelial cells. her2 gene amplification is a crucial proto-oncogene and well established as a prognostic-predictive biomarker in breast cancers (1-4). Former studies have indicated different frequency of Her2 over-expression in breast cancer patients from 18 to 30% (5,6).

It has been reported that the amplification of her2 gene was notable in 10–34% of subjects with invasive breast cancers. Amplification of her2 gene has been identified in cases with progression of the breast cancer or cancer metastasis. Therapy with trastuzumab; a monoclonal antibody to Her2 protein would be effective merely in cases with her2 gene amplification and protein overexpression. Moreover, resistance to each therapy protocols including chemotherapeutic factors or hormonal medications are observed with amplification of her2. Thereby, accurate and consistent assessment of her2 status is a crucial step in the guidance of disease management and treatment (7-9).

Breast cancer prognosis, recurrence, management and response to therapy are strongly associated to the laboratory study of her2 status. Evaluation of the her2 status is performed by different FDA (Food and Drug Administration) approved methods. For instance, cell membrane protein overexpression is assessed by immunohistochemistry (IHC) but this method is unable to determine the chromosomal and genetic alterations. The gene amplification is evaluated by in situ hybridization (CISH) method in equivocal cases. The associations between histopathologic variables, status of Ki-67 index, progesterone and estrogen receptors (PR & ER) with her2 amplification by CISH were investigated and analyzed. The level of significance was considered as P-value < 0.05.

Results: Totally, 239 patients with mean age of 53.2 years were studied. CISH identified her2 gene amplification in 51 subjects (21.3%). The type of tumor (invasive ductal carcinoma), the tumor grade, and the value of Ki-67 index were directly correlated with her2 amplification. Significant negative associations were also observed between CISH results and ER and PR expression.

Conclusion: As her2 gene amplification was identified in 21.3% of invasive breast cancer patients with equivocal IHC results, it is supposed that applying CISH method may consider as a potentially valuable supplementary method. Results have also shown that higher grades of tumor, invasive ductal carcinoma, absences of hormone receptors and high Ki-67 index significantly correlated with the her2 amplification.
hybridization methods like FISH and Polymerase Chain Reaction (PCR) as highly sensitive techniques. In IHC equivocal cases FISH is considered as the gold standard; however, this method is not very practical for routine diagnostic laboratories. FISH technique requires modern and expensive fluorescence microscope with high-quality immersion objectives, filters and recording camera. Analyses of FISH data is time consuming and fluorescence signals can be faded after several weeks (7,10-13). Recently, Chromogenic in situ Hybridization (CISH) Method has been introduced as another technique for determination of the level of her2 gene amplification based on enzymatic reaction. In more than 90% of cases, the results of CISH are compatible with the other diagnostic methods and it can be performed as a check test for patients with score +2 in IHC method. As another important advantage, using chromogens instead of fluorochromes for signal detection could provide a standard bright field microscope. Finally, visualized signals using CISH method do not fade during time and a long-time archives could be accessible (12,14,15).

Among Iranian women, breast cancer is the most frequent malignancy with standard incidence rate of 27.4 per 100,000 populations (16). Determination of her2 gene status is of importance to know about the prognosis and prediction of patient’s response to anti-Her2 monoclonal antibody therapy. In Iran, using CISH method is not routine because of some limitations as well as high price of CISH Kit. It was reported that 11% of patients with invasive primary breast carcinoma had equivocal IHC results (17), so it is supposed that in such cases, applying CISH method may consider as a potentially valuable supplementary tool. In the present study, we aimed to investigate the predicting effects of clinicopathological variables on her2 gene amplification by CISH assay in Her2 (2+) breast cancer subjects. Moreover ER, PR, and Ki-67 status in the cases were evaluated.

Materials and Methods
This cross-sectional study was conducted in Zaferanyeh Laboratory a referral private pathology center collaborated with Shahid Beheshti University of Medical Sciences (Tehran-Iran from 2015 to 2018). Population study included all invasive breast cancer patients with equivocal (2+) results on immunohistochemistry technique (IHC) who were referred for more investigation by chromogenic in situ hybridization (CISH) method. Confirmation of IHC-equivocal (2+) Her2 result (by an expert pathologist) and successful CISH staining were considered as inclusion criteria. Subsequently, availability of archival H&E slides as well as ER, PR, and Ki67 IHC slides of tumors were considered for further investigation. Participants’ clinicodemographic data such as gender, age, type of sample, tumor size and tumor focality were obtained from patients’ medical records. The H&E and IHC slides were reviewed and assessed histopathological data; were histologic tumor types, histologic tumor grade, lymph nodes involvement, vascular invasion, calcifications, carcinoma in situ, status of ER, PR and Ki67 IHC markers. In the next step, CISH slides were interpreted by an expert pathologist and CISH results were recorded in the checklists. In this study CISH technique using Zyto Dot 2C SPEC ERBB2/CEN 17 Probe Kit was performed by trained laboratory technician according to the manufacturer’s instruction (www.Zytovision.com). ERBB2 gene sequencing and genomic organization of alpha satellite DNA on centromeric regions of chromosome 17 were visualized in green and red signals, using a light microscope.

CISH interpretation was performed by objective lens x60. All red and green signals of 20 tumor cells were counted in invasive component of tumor, in high signals region without necrosis or nuclei overlapping. Then the ratio was calculated. The results less than 2 and equal or more than 2 were defined as negative and positive values, respectively. In intermediate results (ratios between 1.8 and 2), 20 other tumor cells were considered, and the ratios were determined in 40 cells.

Finally, associations between her2 gene amplification status using CISH technique and clinicopathological variables in IHC-equivocal (2+) Her2 in invasive breast cancer subjects were statistically evaluated.

The present study was taken from a medical resident thesis with ID: M317. Ethics approval was obtained from the institutional review board of Shahid Beheshti University of Medical Sciences according to Helsinki declaration (IR.SBMU.MSP.REC.1397.139). All gathered data were considered confidential and no extra cost was imposed on our participants.

Data Analysis
Analyses were statistically performed by using SPSS 21 (SPSS Inc., Chicago, Ill., USA). Quantitative and qualitative variables were reported by mean±SD and percent, respectively. Student t test and Chi square were used for comparing quantitative and qualitative variables data. Moreover, the level of significance was considered as P-value<0.05.

Results
Totally, 239 (236 women and 3 men) cases with the mean age of 53.12±12.35 years (Min; 30 & Max; 86) entered the study. The overall frequency of her2 gene amplifications was found in 51 subjects (21.3%), by CISH- positive results. Tissue section H&E slides and IHC markers related to 207 cases including 189 resection and 18 core needle biopsy samples were available.

Descriptive Data Related Subjects with Resection Samples
The mean age of subjects with resection sample was 54.20 ±11.98 years (Min; 32 & Max; 86). The mean size of tumor was 2.47±1.20 cm (Min; 0.50 & Max; 6.5). The most of tumor were unifocal (92.6%). More frequent histologic tumor type was invasive ductal
carcinoma (70.9%) in grade 2 (68.8%). Vascular invasion in 41.3%, lymph nodes involvement in 50% and Carcinoma in situ in 26.5% of cases were demonstrated. Detailed data are shown in Table 1.

ER in 149 patients (78.8%) was positive based on Allred scoring and intensity as follows; 18 (9.5%) weak, 77 (40.7%) moderate and 52 (27.5%) severe. PR in 142 patients (75.1%) was positive based on Allred scoring and intensity as follows; 16 cases (8.5%) weak, 73 (38.6%) moderate and 51 (27%) severe. The mean of Ki-67 was 25.66 ± 16.37. Thirty-nine (20.6%) cases of resection samples demonstrated CISH-positive results.

### Table 1. Characteristics of tumor resection specimens

| Variables                      | Number | Percent |
|-------------------------------|--------|---------|
| **Tumor focality**            |        |         |
| Unifocal                      | 175    | 92.6    |
| Two foci                      | 8      | 4.2     |
| More than two                 | 5      | 2.6     |
| Bilateral                     | 1      | .5      |
| **Tumor grade**               |        |         |
| I                             | 18     | 9.5     |
| II                            | 130    | 68.8    |
| III                           | 41     | 21.7    |
| **Tumor vascular invasion**   |        |         |
| Not identified                | 111    | 58.7    |
| Present                       | 78     | 41.3    |
| **Tumor type**                |        |         |
| Invasive ductal carcinoma     | 134    | 70.9    |
| Invasive lobular carcinoma    | 28     | 14.8    |
| Mixed                         | 20     | 10.6    |
| Others                        | 7      | 3.7     |
| Negative                      | 132    | 69.8    |
| **CIS**                       |        |         |
| DCIS                          | 50     | 26.5    |
| Mixed                         | 2      | 1.1     |
| LCIS                          | 5      | 2.6     |
| **Calcification**             |        |         |
| Absent                        | 157    | 83.1    |
| Present                       | 32     | 16.9    |

**Comparative Data Related Subjects with Resection Samples**

Significant differences were observed between 2 groups (CISH positive vs CISH negative) regarding histologic type of tumor (0.003). Invasive ductal carcinomas in positive CISH group were more frequent compared with negative CISH group. Tumor grades were also significantly different between 2 groups (P=0.017) with direct relationship. The numbers of positive ER and PR samples in negative CISH group were significantly higher than positive CISH group (P<0.001 & P= 0.002). Medians of markers including Ki-67 index, ER & PR percent were also statistically different between two groups P<0.05).

On the other hand, tumor size was not different between groups (2.47±1.22 vs. 2.49±1.13; P=0.937) and no significant difference was found between 2 CISH groups regarding the age of patients (P=0.117). Detailed data regarding the positive and negative CISH groups are demonstrated in Table 2.

### Table 2. Comparing characteristics in positive and negative CISH groups

| Variables | Positive CISH | Negative CISH | P-value |
|-----------|---------------|---------------|---------|
| Age (year)| 51.26±11.39   | 55.29±11.96   | 0.117   |
| Gender    |               |               |         |
| Female    | 39 (20.9)     | 148 (79.1)    |         |
### Effective Variables on Her2 CISH Result

| variables                      | Positive CISH | Negative CISH | P-value |
|--------------------------------|---------------|---------------|---------|
| Male                           | 0             | 2 (100)       | 0.468   |
| Tumor size                     | 2.47±1.22     | 2.49±1.13     | 0.937   |

#### Tumor Type (Count %)

| Tumor Type                      | Count (%)  | Count (%)  |
|---------------------------------|------------|------------|
| Invasive ductal carcinoma       | 33 (24.6)  | 101 (75.4) |
| Invasive lobular carcinoma      | 1 (3.6)    | 27 (96.4)  |
| Mixed                           | 4 (57)     | 19 (95.0)  |
| Other                           | 1 (50)     | 3 (43.0)   |

#### Tumor focality (count %)

| Tumor focality                  | Count (%)  | Count (%)  | P-value |
|---------------------------------|------------|------------|---------|
| unifocal                        | 36 (20.9)  | 136 (79.1) | 0.999   |
| two foci                        | 2 (25)     | 6 (75.0)   |         |
| more than two                   | 1 (20)     | 4 (80)     |         |
| bilateral                       | 0          | 1 (100)    |         |

#### Tumor grade (count %)

| Tumor grade | Count (%) | Count (%) | P-value |
|-------------|-----------|-----------|---------|
| I           | 1 (5.6)   | 17 (94.4) | 0.017   |
| II          | 25 (19.2) | 105 (80.8)|         |
| III         | 13 (31.7) | 28 (68.3) |         |

#### Positive vascular invasion (count %)

| Positive vascular invasion | Count (%) | Count (%) | P-value |
|----------------------------|-----------|-----------|---------|
| negative                   | 25 (19.1) | 106 (80.9)| 0.247   |
| DCIS                       | 14 (28.0) | 36 (72.0) |         |
| mixed                      | 0         | 2 (100)   |         |
| LCIS                       | 0         | 5 (100)   |         |

#### Carcinoma in situ (count %)

| Carcinoma in situ | Count (%) | Count (%) | P-value |
|-------------------|-----------|-----------|---------|
| negative          | 4 (12.5)  | 28 (87.5) | 0.207   |
| DCIS              |           |           |         |
| mixed             |           |           |         |
| LCIS              |           |           |         |

#### Micro calcification (count %)

| Micro calcification | Count (%) | Count (%) | P-value |
|---------------------|-----------|-----------|---------|
| positive ER (count %) | 23 (15.4) | 126 (84.6) | <0.001  |

#### ER intensity (count %)

| ER intensity | Count (%) | Count (%) | P-value |
|--------------|-----------|-----------|---------|
| Mild         | 5 (27.8)  | 13 (72.2) | 0.541   |
| Moderate     | 10 (13.0) | 67 (87)   |         |
| Severe       | 8 (15.4)  | 44 (84.6) |         |

#### Positive PR (count %)

| Positive PR | Count (%) | Count (%) | P-value |
|-------------|-----------|-----------|---------|
| weak        | 4 (25.0)  | 12 (75.0) | 0.430   |
| Moderate    | 11 (15.1) | 62 (84.9) |         |
| Severe      | 7 (13.7)  | 44 (86.3) |         |

#### PR intensity (count %)

| PR intensity | Count (%) | Count (%) | P-value |
|--------------|-----------|-----------|---------|
| weak         | 4 (25.0)  | 12 (75.0) |         |
| Moderate     | 11 (15.1) | 62 (84.9) |         |
| Severe       | 7 (13.7)  | 44 (86.3) |         |

#### Markers; Median (IQR)

| Markers; Median (IQR) | Count (%) | Count (%) | P-value |
|-----------------------|-----------|-----------|---------|
| Number of involved node | 0 (2)     | 0 (2)     | 0.860   |
| ER percent            | 5 (60)    | 80 (50)   | <0.001  |
| PR percent            | 3 (30)    | 60 (80)   | <0.0001 |
| Ki-67                 | 30 (24)   | 19 (23)   | 0.010   |

#### Number of involved lymph node (count %)

| Number of involved LN | Count (%) | Count (%) | P-value |
|-----------------------|-----------|-----------|---------|
| 1-3                   | 11 (26.2) | 31 (73.8) | 0.421   |
| 4-9                   | 2 (8.7)   | 21 (91.3) |         |
| >9                    | 3 (27.3)  | 8 (72.7)  |         |

#### Tumor size (count %)

| Tumor size | Count (%) | Count (%) | P-value |
|------------|-----------|-----------|---------|
| <2 cm      | 17 (18.1) | 77 (81.9) | 0.415   |
| 2-5 cm     | 22 (25.6) | 64 (74.4) |         |
| >5 cm      | 0         | 5         |         |
Descriptive data related subjects with fine needle biopsies

Of all included participants, 18 patients had fine needle biopsies. The mean age of subjects was 46 ± 12.39 years (Min; 31 & Max; 80). Nobody had tumor grade I while the most cases (77.8%) had tumor grade II. Vascular invasion in 11.1% of specimens was observed and type of tumor in 88.9% was invasive ductal carcinoma. DCIS and calcification in 11.1% and 5.6% were demonstrated. Detailed data are shown in Table 3.

ER in 10 patients (55.6%) was positive based on Allred scoring and intensity as follow; 2 cases weak, 6 moderate and 2 severe. PR in 12 patients (66.7%) was positive based on Allred scoring and intensity as follow; 3 cases weak, 5 moderate and 4 severe. The mean of Ki-67 was 38.31 ± 19.94.

| Variables                     | Number | Percent |
|-------------------------------|--------|---------|
| Tumor grade                   |        |         |
| I                             | 0      | 0       |
| II                            | 14     | 77.8    |
| III                           | 4      | 22.2    |
| Tumor vascular invasion       |        |         |
| Not identified                | 16     | 88.9    |
| Present                       | 2      | 11.1    |
| Tumor type                    |        |         |
| Invasive ductal Carcinoma     | 16     | 88.9    |
| Invasive lobular Carcinoma    | 0      | 0       |
| Mixed                         | 0      | 0       |
| Others                        | 2      | 11.1    |
| CIS                           |        |         |
| Negative                      | 16     | 88.9    |
| Present                       | 2      | 11.1    |
| Mixed                         | 0      | 0       |
| LCIS                          | 0      | 0       |
| Calcification                 |        |         |
| Absent                        | 17     | 94.4    |
| Present                       | 1      | 5.6     |

Comparative Data Related Subjects with Fine Needle Biopsies

Detailed data regarding the positive and negative CISH groups are demonstrated in Table 4. The mean ages in patients with positive and negative CISH were 44.33±9.29 and 46.33±13.16 years. No significant difference was found between 2 positive and negative CISH groups in regard with age of patients (P=0.708). Significant differences were not observed between 2 groups regarding the histologic type of tumor (P=0.314). Grades of tumor, presence of vascular invasion and carcinoma in situ were not significantly different between the groups (P=0.999, P=0.313 & P=0.999, respectively). Nobody in positive CISH group and one case in negative CISH group showed calcification (P=0.999). The numbers of positive ER and PR in negative CISH group were higher than positive CISH group; however, differences were not statistically significant (P=0.537 & P=0.515). Medians of markers including Ki-67 index, ER & PR percent were not also statistically different between two groups (P<0.05).

| Variables                     | Positive CISH | Negative CISH | P-value |
|-------------------------------|---------------|---------------|---------|
| Age (year)                    | 44.33±9.29    | 46.33±13.16   | 0.708   |
| Tumor Type (Count %)          |               |               |         |
| Invasive ductal carcinoma     | 2 (12.5)      | 14 (87.5)     |         |
| Invasive lobular carcinoma    | 0             | 0             |         |
| Invasive medullary carcinoma  | 0             | 0             | 0.314   |
| Other                         | 1 (50)        | 1 (50)        |         |
### Discussion

Evaluation of Her2 protein overexpression by IHC method is routinely used to determine prognosis and therapeutic responsiveness in cases with invasive breast cancer. This technique can be easily accessed and it is not expensive compared to in situ hybridization methods or PCR. Nevertheless in cases with equivocal IHC results, using other evaluating methods seems inevitable. In situ hybridization methods are being performed in very limited Iranian laboratories due to high expenses, not being covered by insurance companies and lack of laboratory facilities. On the other hand, increasing trend of breast malignancies rates in our country necessitates more available laboratory techniques with high sensitivity and accuracy. Therefore, the present study has been conducted to determine statistical data related CISH results detecting her2 gene amplification in invasive breast cancer patients with equivocal IHC results. Moreover, associations between some biomarkers and clinicopathological criteria with CISH results were also assessed. Determination of validity of such clinical data correlated to CISH results may provide a valuable index in patient selection for using other supplementary methods.

According to our results, frequency of her2 gene amplifications in CISH- positive group was 21.3%; including resections (20.6%) and needle biopsies (16.7%). This rate shows that about one-fifth of invasive breast cancer cases with equivocal IHC results need more supplementary investigations. Consistent to our results, Zhao et al. demonstrated a near frequency rate by 19% (7). Mehrzama et al. have also indicated that of 201 equivocal IHC cases, her2 gene amplification by CISH was observed in 42 patients (20.9%) (kappa: 0.42). In investigations by Lan et al., Wolff et al. and Alsaﬁ et al., this rate was reported 24 and 44 and 44.4%, respectively (18–20). Overall, it is supposed that the rate of her2 gene amplification by CISH in equivocal IHC cases vary from 19 to 44%.

Among resection biopsies, most of them showed unifocal, grade II, no vascular invasion, and invasive ductal carcinoma form. Like resection biopsies, grade II, invasive ductal carcinoma, and no vascular invasion were the most frequent characteristics in samples by needle biopsy.

According to the results, a positive association was observed between tumor type and her2 amplification; frequency of invasive ductal carcinoma in resection biopsies with positive CISH group was higher compared to negative CISH group (P=0.003). Prior studies have also confirmed this relationship between her2 amplification and histopathologic forms of ductal carcinoma. It is supposed that her2 amplification defines a group of ductal carcinoma cases with greater invasive potential which need more aggressive therapy (21).
Compatible to our finding, Foruhesh Tehrani et al. have indicated a significant difference between frequency of invasive ductal carcinoma in positive and negative CISH groups (100% vs. 76.5%; P=0.026) (22). Karegar et al. demonstrated a higher frequency of ductal carcinoma diagnosis in positive Her-2 overexpression in comparison with negative Her-2 overexpression patients; however, the difference was not statistically significant (52.8 vs. 47.2%; P=0.77) (23). Taghipour et al. also could not demonstrate a meaningful relationship between the type of tumor and Her2/neu overexpression (24).

Comparing characteristics in resection group with positive CISH results, grade 2 was the most prevalent (25 cases; 19.2%) and also a positive association was found between Her2 overexpression with higher grades of tumor (P=0.017). Consistent to our result, Taghipour et al. have revealed both the most prevalent tumor grade 2 as well as a meaningful correlation between the Her2/neu overexpression and grade of tumor (P=0.002) (24). Alsafi et al. have also shown a significant relationship between her2 gene amplification and the higher grade of tumor (P=0.05) (20).

Regarding hormone receptors in resection group, estrogen receptor in 78.8% was positive including 31.2% moderate and 27.5% severe intensity. Progesterone receptor in 75.11% was also positive as follows: 28.6% with moderate and 27% with severe intensity. Analyses of data demonstrated that in resection group, positive CISH results in positive ER were significantly lower than in negative ER (15.4% vs. 43.9% P<0.001). This significant difference was also observed regarding the progesterone receptors (15.5% vs. 37%; P=0.002). On the other hand, no significant associations were found between positive CISH and intensities of both receptors. In accordance with our findings, Zaidoon et al. reported a negative significant relationship between both estrogen and progesterone receptors with her2 amplification (P=0.002 and 0.017, respectively) (8). These findings related her2 gene status with negative ER and PR were also confirmed in 182 invasive breast cancer patients with equivocal IHC by Ji et al. (P<0.001) (9).

Based on the results of present study, the median of Ki-67 in resection biopsies was 25.66%. This marker between positive and negative CISH groups was also statistically different; Ki-67 index was significantly expressed in positive CISH groups (P=0.010). Other investigations by Ji et al. and Taghipour et al. have demonstrated a significant association between increase of Ki-67 index and her2 amplification (P=0.006 and P=0.03, respectively) (9,24). On the other hand, Alsafi et al. have not found this significant association between her2/neu gene amplification and ki-67 index (20).

Our study had some limitations. First, the number of samples was limited particularly in needle biopsy group. This limitation could affect validity and reliability of our findings. Second, our results were based on patients from one center and the results cannot be generalized. For better outcome we recommend a bigger sample from different centers.

**Conclusion**

Results obtained by CISH method indicated that her2 gene amplification was identified in 21.3% of invasive breast cancer patients with equivocal IHC results. There were significant associations between her2 gene amplification with some clinicopathologic variables in equivocal IHC cases. Higher grades of tumor, invasive ductal carcinoma, absences of hormone receptors and high Ki-67 index significantly correlated with the her2 amplification. In equivocal IHC cases, mentioned above clinicopathological variables may consider as a potentially valuable predicting factors on her2 gene amplification status, in spite of some limitations related using CISH method in our country.

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**Conflict of Interest**

The authors declared that there is no conflict of interest regarding the publication of this article.

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