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Fluorescent in Situ Hybridization and Real-Time Quantitative Polymerase Chain Reaction to Evaluate *HER-2/neu* Status in Breast Cancer

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KEYWORDS

Breast Cancer
HER-2/neu
Gene Quantification
Quantitative Real-Time PCR

ABSTRACT

Background: Breast cancer remains the most common and second lethal cancer in females. *HER-2/neu* is one of the most important amplified oncogene in breast cancer. The amplification of *HER-2* is correlated with decreased survival, metastasis, and early recurrence. The amplification of *HER-2/neu* gene and synthesis of the protein are reported in 10%-34% of breast cancer cases associated with tumor size, advanced tumor stage, high-grade tumor, young age at diagnosis, absence of steroid hormone receptor, and lymph node involvement.

***Methods*:** Fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC) methods are options to evaluate *HER-2* expression. The current study aimed at identifying the correlation between FISH and real-time polymerase chain reaction (PCR) in measuring *HER-2* expression.

***Results*:** The study investigated the performance of the real-time PCR as measured against FISH method in IHC +2 borderline cases. In a total of 120 IHC 2+ samples, 58.3% were negative and 41.6% positive for *HER-2* gene, confirmed by FISH as a gold standard method. The real-time PCR ratio was <1.8 for a majority (82.8%) of the tumor samples with unamplified *HER-2* gene by FISH as a gold standard assay.

***Conclusion*:** Despite the fact that real-time PCR is a promising method to evaluate *HER-2* over expression and a supplementary array to FISH, according to the results of the present study it cannot be utilized instead of gold standard techniques; therefore, additional studies should be carried out to appraise the value of this method to evaluate *HER-2* over expression.

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Introduction

Breast cancer remains the most common and second lethal cancer in females (1). It occurs in Iranian females at least one decade earlier than their Western counterparts, and the incidence rate is 120 cases per 100 000 females in Iran (2). Etiology of the breast cancer is multifactorial by genetics, environmental, and reproductive factors interacting in an intricate path. *HER-2/neu* is one of the most important amplified oncogenes in breast cancer (3). This gene is located on chromosome 17q12 and encodes a 185-kDa transmembrane glyco-phosphoprotein with tyrosine kinase activity (3, 4) .The incidence of *HER-2* gene amplification and its prognostic value in breast cancer make *HER-2* an exciting target to improve therapeutic agents (5). Also, it is a useful marker to predict the response of patients to chemotherapy, hormonal therapy, and therapeutic anti-HER-2 antibodies (6). Trastuzumab (Herceptin) is a humanized monoclonal antibody,

specifically against the extracellular domain of HER-2 protein, which is a new drug for target therapy of patients with *HER-2* positive (7). Fluorescent in situ hybridization (FISH) is one of the approved methods (8, 9). Amplification of *HER-2* gene by FISH assay showed a specificity of 100% and sensitivity of 98%. Quantitative polymerase chain reaction (PCR) is a facile, rapid, and independent of particular reagents such as antibodies with a high level of sensitivity and precision (10). The current study aimed at evaluating the value of real-time PCR method in primary breast cancer and comparing the results with FISH analysis to discern the status of *HER-2/neu* oncogene at DNA, RNA, and protein levels.

Materials and Methods

The current study examined samples of formalin-fixed paraffin embedded (FFPE) breast carcinomas obtained from the molecular pathology department of Ghaem Hospital, Mashhad, Iran. Samples were obtained from 120 patients with early stage of breast cancer from 2012 to 2013. The samples were the routine surgical specimens fixed in formalin, according to standard histological protocols. Blocks of paraffin–embedded tumor tissue from patients were used to prepare 5 µm section for FISH. For real-time PCR, sections containing >80% tumor cells were selected for DNA extraction.

DNA preparation

On the slides of each sample, an area containing tumor cells was selected and marked by a pathologist; thereafter, 2 tissue microarray sample cores with 0.9 mm in diameter were obtained from the selected area in each paraffin block. DNAs were extracted using QIAamp DNA FFPE Tissue Kit (Qiagen, Germany Cat No: 56404), according to the QIAGEN protocol. The quality and concentration of the DNAs were defined using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Immunohistochemistry

IHC is the most frequently used assay that can be performed on paraffin-embedded or frozen tumor sample, and also used to access the overexpression of *HER-2* gene; it is a widely applied method to measure gene amplification (11). IHC is a semi-quantitative scale of positive and negative predictive values varied by stain intensity, ranging from 0 to 3+ (2, 11). Outcome of tumor staining that demonstrate the moderate gene expression (2+) are considered ambiguous consequences and should be evaluated for HER2 gene amplification with hybridization methods such as FISH analysis. IHC staining for *HER-2* had been performed on 120 FFPE tumor samples, and the Hercep Test was applied (Dako, Glostrup, Denmark). The test was performed exactly according to the manufacturer's instructions. The results of Hercep Test IHC were scored as 0, no staining or membrane staining in <10% of cells; 1+, weak or barely perceptible staining in >10% of cells, and only part of the membrane in > 10% of cells; 2+, weak to moderate staining of the entire membrane in >10% of cells; 3+, strong staining of the entire membrane in >10% of cells.

Real-time qPCR

Detection of *HER-2* gene and the reference gene *IGF-1* were conducted by real-time qPCR method in the Applied Biosystems® StepOne™. All reactions were run in duplicate in separate wells and contained 10 µL mixture; each reaction contained 0.5 g/L bovine serum albumin, 6 mM $Mgcl_2$, 0.5 µM of each primer, 0.2 µM of each hybridization probe, 0.2 mM oxynucleotide triphosphate, and 0.5 U Taq DNA polymerase in 1X PCR buffer, and 2 µL of DNA extract with a concentration of 4 ng. The PCR program started with 1 cycle at 95ºC for 30 seconds, followed by 50 amplification cycles at 95ºC for 3 seconds, 55ºC for 5 seconds, and 72ºC for 10 seconds .The 5′ and 3′ end nucleotides of the probe were labeled with the reporter FAM (6-carboxy-fluorescein) and the quencher dye TAMRA (6-carboxy-tetramethylrhodamine), respectively. All reactions were performed in an ABI PRISM® 7700 Sequence Detection System (Applied Biosystems AB, USA). The primers and probes used in the analysis are presented in Table 1.The content of the target in tumor samples was quantified by standard curves to determine a relative measure of the starting amount. The absolute target copy numbers were resolved with utilization of 1:2 dilution series of

genomic DNA as a standard control gene. For each clinical sample, the amounts of the target gene (*HER-2/neu*) and the reference gene (*IGF-1*) were calculated in tumor and healthy control tissue. Complete instruction was described previously (4), a cut off value was the ratio of 1.8; it means that a sample with a ratio of < 2 was considered negative and a sample with a ratio of > 2 was regarded positive (Figure1).

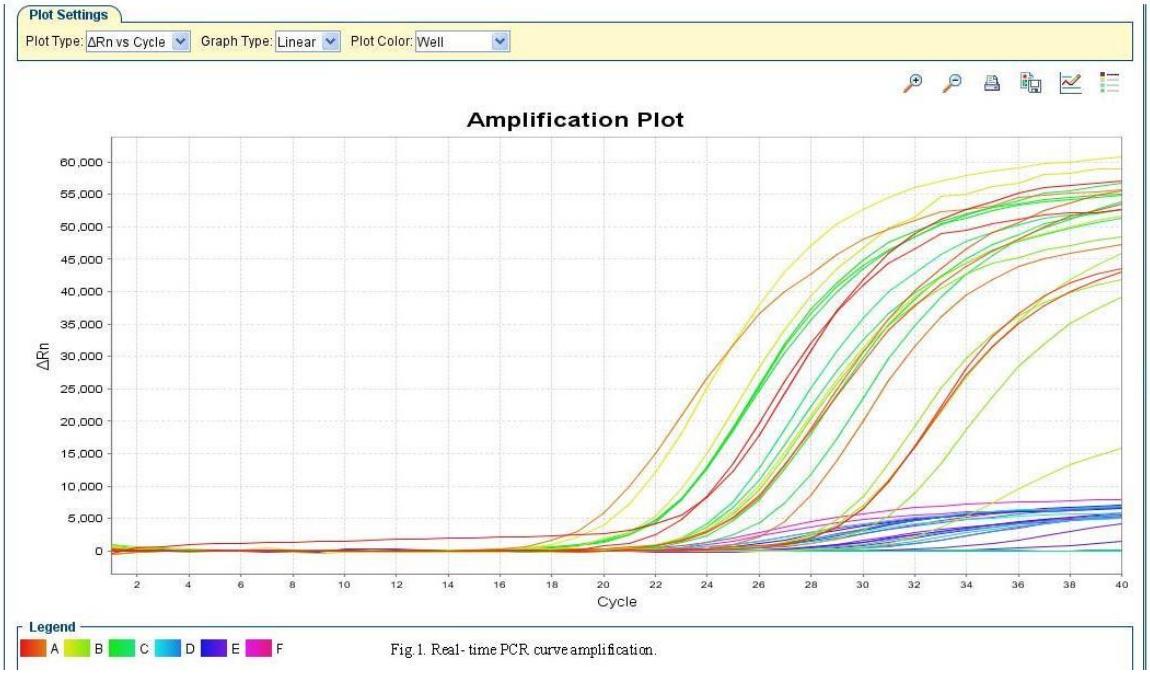

Fig. 1. Real-Time PCR curve amplification.

Florescence in situ hybridization

FISH was performed on 3 µm thick sections of FFPE tissue using *HER-2/neu* (spectrum green) / CEP 17 (spectrum red) DNA probe (Zytovision Gmbh, Fischkai, Germany) as recommended by the manufacture. The number of red and green signals was counted in a minimum of 100 tumor cells nuclei in each section. HER-2/CEP17 ratio score of ≤ 2.2 was classified as unamplified and a score of > 2.2 as amplified

Statistics

The relationships between grouped variables were analyzed by the Fisher exact test. All P-values <0.05 were considered statistically significant.

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Table. 1. Sequence of Probes and Primers Used in the Study

Target	Name[a]	Sequence , 5´ to 3´
HER-2/neu	neu-F	GAACTGGTGTATGCAGATTGC
	neu-R	AGCAAGAGTCCCCATCCTA
Probe	neu-up	GTATGCACCTGGGCTCTTTGCAGGTCTCT-FAM
	neu-down	LCRed640 CCGGAGCAAACCCCTATGTCCACAAGG-p
IGF-1	IGF-F	AGCTCGGCATAGTCTT
	IGF-R	CCAAGTGAGGGGTGTGA
Probe	IGF-up	ATGAGACAGTGCCCTAAAGGGACCAATCCAATG-FAM
	IGF-down	LCRed640 CTGCCTGCCCCTCCATAGGTTCTAGGAAATGAG

Results

Since FISH is the gold standard assay to evaluate *HER-2* gene amplification, the study investigated the performance of the real-time PCR, compares with that of the FISH method in IHC +2 borderline cases. The status of *HER-2* on 120 primary tumors ranged from 0.8 to 16.3, based on real-time PCR. According to the other studies and the manual, a reasonable cut off value was the ratio of 1.8, it meant that a sample with a ratio of <1.8 was considered

negative and a sample with a ratio of >1.8 was regarded positive. And HER-2/CEP17 ratio score of ≤ 2.2 was classified as unamplified and a score of >2.2 as amplified by FISH assay. In a total of 120 IHC 2+ samples, 58.3% were negative, and 41.6% positive for *HER-2* gene amplification confirmed by FISH as the gold standard method. The real-time PCR ratio was <1.8 for a majority (82.8 %) of the tumor samples with unamplified *HER-2* gene by FISH as the gold standard assay. Discrepancies were found in 12 samples that were positive by FISH and negative by real-time PCR. The summary of the results is illustrated in Figure2.

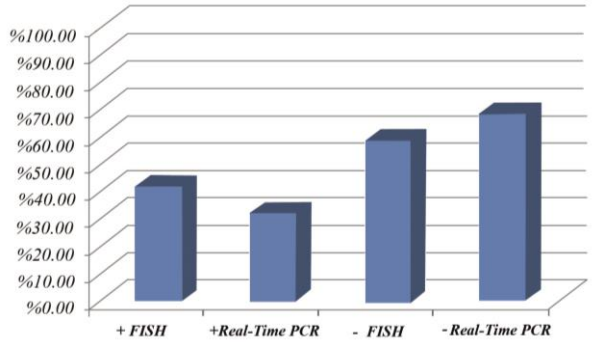

Fig 2. Results of positive /negative samples for FISH and Real-Time PCR.

Discussion

In Expression of *HER-2/neu* is observed in 10%-35% of the human breast carcinomas (12). Therefore, it is necessary to determine treatment pathways for patients with *HER-2* gene amplification that may be associated with variant effects of anti-hormonal therapy (13). According to ASCO/CAP criteria, amplification of *HER-2* gene is defined as membranous HER-2 protein synthesis in >30% of tumor cells (14).Treatment of patients with breast carcinomas by consuming trastuzumb is based on the *HER-2* status of tumor cells (15). Several methods can be performed to determine the *HER-2* gene amplification or HER-2 protein synthesis including florescent in situ hybridization (FISH), immunohistochemistry (IHC) (16), semi-quantitative methods such as chromogenic in situ hybridization (CISH) (3) and quantitative measurement of *HER-2/neu* DNA/RNA by qPCR (17). The current study determined the *HER-2/neu* expression using IHC as an initial screening test and selected only IHC +2 samples to compare - *HER-2/neu* gene amplification by FISH and real-time PCR methods to appraise the feasibility to use real-time PCR to the specified *HER-2* status in FFPE breast tumors. A 90% concordance was found between the results of real-time PCR and FISH. By comparison, Olsson et al., (15) found 93% agreement between real-time PCR and FISH in a study, Wang et al., (7) 92.1%, and Gjerdrum et al., (18) reported the confirming rate of 83% in the analysis of micro-dissected tumors and suggested the use of real-time PCR as a supplement to FISH and IHC. Nistor et al., (17) conducted a similar study on borderline IHC +2 category and compared FISH and real-time PCR methods, and found 92% concordance. Barberis et al., determined that Q-RT-PCR was a fast, sensitive, reliable, and cost-effective technique to estimate *Her-2* status in frozen and FFPE breast cancer samples, which could be applied to regular clinical practices (8). Tvrdík et al., (19) showed that FISH, IHC, and qPCR are highly comparable to detect *HER-2* amplification and over-expression. Other studies showed high agreement between FISH and real-time PCR (6, 20, 21). The experiment performed by Dressler et al., showed that none of the FISH, PCR, and IHC can better predict the outcome of tumor therapy with cyclophosphamide, doxorubicin, and fluorouracil (22). Real-time PCR is a rapid, cost-effective, and reproducible method; one of the problems in real-time PCR is the presence of non-malignant cells in tumor samples, which leads to much smaller amplified gene products compared to the results when the factor did not exist (23); the problem can be solved by applying laser capture micro-dissection to separate tumor cells. A study demonstrated that the dilution of malignant cells with normal cells was not a serious problem to determine *HER-2* status (18). Luoh et al., demonstrated that a subset of human breast cancers that were positive for *HER-2* gene amplification with FISH method did not perform HER-2 protein synthesis; therefore, it was important to use supplemental methods such as RT-PCR to identify the overexpression of *HER-2*, since the first group does not benefit from anti-HER2 therapy (24). IHC is widely accessible and detects over expression of the HER-2/neu and can be perform at a reasonable cost (25). In a study on

patients with IHC 2+ , using CISH method, the amplification of *HER-2* was about 34% of 1397 tumors; also, 16 studies showed amplification in more than 60% of IHC 2+ tumors (26). Another experiment showed that IHC 2+ tumors without *HER-2* gene amplification could indicate that some of these cases were over-stained or over-read in IHC analysis. Tumor heterogeneity was another reason for these findings; the study reported that 3% of samples showed heterogeneous IHC staining and hybridization signals (27). Heterogeneity was also detected by FISH in IHC 2+ tumors and showed low-level amplification in some areas and mixture of high- and non-amplification in other areas (28). Barrett et al., investigated 153 equivocal *HER-2* IHC 2+ by FISH method and showed that 19% of patients had amplification of the *HER-2* gene (29). Chibon et al., selected a group of 108 *HER-2* IHC 2+ invasive patients with breast cancer and the analysis by FISH showed amplification of *HER-2* gene in 24% of patients (24); while, Panjwani et al., showed that 66.6% of IHC 2+ cases had *HER-2* gene amplification by FISH as gold standard and mentioned that it is necessary to use this method to determine *HER-2/neu* status in IHC equivocal cases (30). The current study results showed that between 120 borderline tumor samples only 41.6% had *HER-2* gene amplification and 58.3% were negative.

Conclusion

Despite the fact that real-time qPCR is a promising method to detect *HER-2* overexpression and as a supplementary array to FISH, according to the results of the present study it cannot be utilized instead of gold standard techniques; therefore, additional studies should be performed to evaluate the value of this method in detection of *HER-2* overexpression.

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Conflict of Interest: The authors declare that there was no conflict of interest.

References

1. Park S, Wang HY, Kim S, Ahn S, Lee D, Cho Y, et al. Quantitative RT-PCR assay of HER2 mRNA expression in formalin-fixed and paraffin-embedded breast cancer tissues. International journal of clinical and experimental pathology. 2014;7(10):6752-9.

2. Hojati Z, Orangi E. HER-2/neu gene amplification assessment in breast cancer patients in Isfahan province by real time PCR, differential PCR and immunohistochemistry. Gene. 2012;497(2):237-42.

3. Abdul Murad NA, Razak ZA, Hussain RM, Syed Hussain SN, Ko Ching Huat C, Che Md Ali SA, et al. Quantification of Her-2/Neu gene in breast cancer patients using real time-polymerase chain reaction (Q-PCR) and correlation with immunohistochemistry findings. Asian Pacific journal of cancer prevention: APJCP. 2013;14(3):1655-9.

4. Königshoff M, Wilhelm J, Bohle RM, Pingoud A, Hahn M. HER-2/neu gene copy number quantified by real-time PCR: comparison of gene amplification, heterozygosity, and immunohistochemical status in breast cancer tissue. Clin Chem. 2003; 49(2):219-29.

5. Tanioka M, Sasaki M, Shimomura A, Fujishima M, Doi M, Matsuura K, et al. Pathologic complete response after neoadjuvant chemotherapy in HER2-overexpressing breast cancer according to hormonal receptor status. Breast (Edinburgh, Scotland). 2014; 23(4):466-72.

6. Benohr P, Henkel V, Speer R, Vogel U, Sotlar K, Aydeniz B, et al. Her-2/neu expression in breast cancer--A comparison of different diagnostic methods. Anticancer research. 2005; 25(3):1895-900.

7. Wang H-y, Kim S, Park S, Kim S, Jung D, Park KH, et al. Evaluation of a quantitative RT-PCR assay to detect HER2 mRNA overexpression for

diagnosis and selection of trastuzumab therapy in breast cancer tissue samples. Experimental and Molecular Pathology. 2014;97(3):368-74.

8. Barberis M, Pellegrini C, Cannone M, Arizzi C, Coggi G, Bosari S. Quantitative PCR and HER2 testing in breast cancer: A technical and cost-effectiveness analysis. American journal of clinical pathology. 2008;129(4):563-70.

9. Shah S, Chen B. Testing for HER2 in Breast Cancer: A Continuing Evolution. Pathology research international. 2011;2011:903202.

10. Perez-Diez A, Morgun A, Shulzhenko N. Microarrays for cancer diagnosis and classification. Advances in experimental medicine and biology. 2007;593:74-85.

11. Kulka J, Tokés AM, Kaposi-Novák P, Udvarhelyi N, Keller A, Schaff Z. Detection of HER-2/neu gene amplification in breast carcinomas using quantitative real-time PCR - A comparison with immunohistochemical and FISH results. Pathology and Oncology Research. 2006;12(4):197-204.

12. Goud KI, Dayakar S, Vijayalaxmi K, Babu SJ, Reddy PV. Evaluation of HER-2/neu status in breast cancer specimens using immunohistochemistry (IHC) & fluorescence in-situ hybridization (FISH) assay. The Indian journal of medical research. 2012;135:312-7.

13. Fehm T, Becker S, Duerr-Stoerzer S, Sotlar K, Mueller V, Wallwiener D, et al. Determination of HER2 status using both serum HER2 levels and circulating tumor cells in patients with recurrent breast cancer whose primary tumor was HER2 negative or of unknown HER2 status. Breast cancer research : BCR. 2007;9(5):R74.

14. Ohlschlegel C, Kradolfer D, Hell M, Jochum W. Comparison of automated and manual FISH for evaluation of HER2 gene status on breast carcinoma core biopsies. BMC clinical pathology. 2013;13:13.

15. Olsson H, Jansson A, Holmlund B, Gunnarsson C. Methods for evaluating HER2 status in breast cancer: comparison of IHC, FISH, and real-time PCR analysis of formalin-fixed paraffin-embedded tissue. Pathology and Laboratory Medicine International. 2013;2013(5):31-7.

16. Schaller G, Evers K, Papadopoulos S, Ebert A, Bühler H. Current use of HER2 tests. Annals of oncology. 2001;12(1): 97-100.

17. Nistor A, Watson PH, Pettigrew N, Tabiti K, Dawson A, Myal Y. Real-time PCR complements immunohistochemistry in the determination of HER-2/neu status in breast cancer. BMC clinical pathology. 2006;6:2.

18. Gjerdrum LM, Sorensen BS, Kjeldsen E, Sorensen FB, Nexo E, Hamilton-Dutoit S. Real-time quantitative PCR of microdissected paraffin-embedded breast carcinoma: an alternative method for HER-2/neu analysis. The Journal of molecular diagnostics : JMD. 2004;6(1):42-51.

19. Tvrdík D, Stanìk L, Skálová H, Dundr P, Velenská Z, Povýšil C. Comparison of the IHC, FISH, SISH and qPCR methods for the molecular diagnosis of breast cancer. Molecular Medicine Reports. 2012;6(2):439-43.

20. Merkelbach-Bruse S, Wardelmann E, Behrens P, Losen I, Buettner R, Friedrichs N. Current diagnostic methods of HER-2/neu detection in breast cancer with special regard to real-time PCR. The American journal of surgical pathology. 2003;27(12):1565-70.

21. Nathanson DR, Nash GM, Chen B, Gerald W, Paty PB. Detection of HER-2/neu gene amplification in breast cancer using a novel polymerase chain reaction/ligase detection reaction technique. Journal of the American College of Surgeons. 2003;197(3):419-25.

22. Dressler LG, Berry DA, Broadwater G, Cowan D, Cox K, Griffin S, et al. Comparison of HER2 status by fluorescence in situ hybridization and immunohistochemistry to predict benefit from dose escalation of adjuvant doxorubicin-based therapy in node-positive breast cancer patients. Journal of Clinical Oncology. 2005;23(19):4287-97.

23. Dandachi N, Dietze O, Hauser-Kronberger C. Evaluation of the clinical significance of HER2 amplification by chromogenic in situ hybridisation

in patients with primary breast cancer. Anticancer research. 2004;24(4):2401-6.

24. Luoh SW, Ramsey B, Hanlon Newell A, Troxell M, Hu Z, Chin K, et al. HER-2 gene amplification in human breast cancer without concurrent HER-2 over-expression. SpringerPlus. 2013;2:386.

25. Madrid MA, Lo RW. Chromogenic in situ hybridization (CISH): a novel alternative in screening archival breast cancer tissue samples for HER-2/neu status. Breast cancer research: BCR. 2004;6(5):R593-600.

26. Rosa FE, Santos RM, Rogatto SR, Domingues MA. Chromogenic in situ hybridization compared with other approaches to evaluate HER2/neu status in breast carcinomas. Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas/ Sociedade Brasileira de Biofisica [et al]. 2013;46 (3):207-16.

27. Kostopoulou E, Vageli D, Kaisaridou D, Nakou M, Netsika M, Vladica N, et al. Comparative evaluation of non-informative HER-2 immunoreactions (2+) in breast carcinomas with FISH, CISH and QRT-PCR. Breast (Edinburgh, Scotland). 2007;16(6):615-24.

28. Lewis JT, Ketterling RP, Halling KC, Reynolds C, Jenkins RB, Visscher DW. Analysis of intratumoral heterogeneity and amplification status in breast carcinomas with equivocal (2+) HER-2 immunostaining. American journal of clinical pathology. 2005;124(2):273-81.

29. Barrett C, Magee H, O'Toole D, Daly S, Jeffers M. Amplification of the HER2 gene in breast cancers testing 2+ weak positive by HercepTest immunohistochemistry: false-positive or false-negative immunohistochemistry? Journal of clinical pathology. 2007; 60(6):690-3.

30. Panjwani P, Epari S, Karpate A, Shirsat H, Rajsekharan P, Basak R, et al. Assessment of HER-2/neu status in breast cancer using fluorescence in situ hybridization & immunohistochemistry: Experience of a tertiary cancer referral centre in India. 2010.

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