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Evaluation of Immunohistochemistry and Silver-Enhanced In Situ Hybridization Results for HER2/neu Manually and with Image Analysis System in Human Breast Cancer

İnsan Meme Kanserinde HER2/neu İmmünohistokimya ve İn Situ Hibridizasyon Sonuçlarının Manuel Olarak ve Görüntü Analiz Sistemi ile Değerlendirilmesi

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ABSTRACT

Objective: HER2/neu (ErbB2) gene status is one of the important information while planing terapy in breast carcinoma. For HER2/neu testing there is not standart assay that has been agreed on. Silver enhanced in situ hybridization is a cantitative and highly reproducible assay. Immunohistochemistry is a cheap and easy assay that has disadvantage of being less reproducible. Recently developed pathologist assisted computerized image analysis systems decrease the ratio of subjectivity due to manual evaluation, enable tele-consultation and make it easy to evaluate tumor morphology and markers. Our aim is to investigate the consistency of manual and computerized interpretation of the results of immunohistochemistry and silver enhanced in situ hybridization.

Material and Method: Immunohistochemisty and silver enhanced in situ hybridization of 73 invasive breast carcinoma results were evaluated manually to determine HER2/neu status. Later, silver enhanced in situ hybridization and immunohistochemistry results were reevaluated with Ventana Image Analysis System. Afterwards correlation of both methods with image analysis system and manuel interpretation were calculated.

Result: All cases were score 2 with immunohistochemistry. With image analysis system, 5 cases were score 1, 56 cases were score 2 and 12 cases were score 3. When in situ hybridization results were reevaluated with image analysis system, 6 cases were discordant compared with manual interpretation.

Conclusion: The correlation rate of immunohistochemistry interpretation results between manuel method and image analysis system was %76; but silver enhanced in situ hybridization interpretation results between manuel method and image analysis system were %91 concordant and it was statistically significant (k= 0.832 and p<0.001).

Key Words: Immunohistochemistry, In situ hybridization, Computer-assisted image analysis, Breast cancer, HER2/neu

ÖZ

Amaç: Meme kanserinde, HER2/neu (ErbB2) gen durumu tedavi planlanmasını yönlendirecek verilerden biridir. HER2/neu durumunu tespit etmek için üzerinde anlaşmaya varılan standart bir yöntem yoktur. Silver enhanced in situ hibridizasyon yöntemi, kantitatif bir yöntemdir ve değerlendiriciler arasındaki uyum yüksektir. İmmünohistokimya, kolay ve ucuz ve kolay bir test olup en önemli dejavantajı değerlendiriciler arasındaki uyumsuzluktur. Son yıllarda geliştirilen patoloğun asiste ettiği bilgisayar aracılı görüntü analiz sistemleri manuel değerlendirmeden kaynaklanan subjektifliği giderebilir; tele-konsültasyona olanak tanır ve tümör morfolojisi ile tümör belirteçlerinin doğru değerlendirilmesini sağlayabilir. Amacımız, in situ hibridizasyon ve immünhistokimya sonuçlarının, manuel değerlendirilmesi ile bilgisayar aracılı değerlendirilmesi arasındaki uyumu araştırmaktır.

Gereç ve Yöntem: 73 adet meme tümörünün, HER2/neu durumunu immünohistokimya ve in situ hibridizasyon ile manuel olarak değerlendirdik. Daha sonra bu olguların immünohistokimya ve in situ hibridizasyon sonuçları Ventana Görüntü Analiz Sistemi ile değerlendirildi. Sonrasında ise her iki yöntemin manuel ve bilgisayar aracılı görüntü analiz sistemi ile değerlendirilme sonuçları arasındaki uyumu araştırıldı.

Bulgular: Tüm olgular immünhistokimya ile skor 2 olarak değerlendirildi. Bu olgular görüntü analiz sistemi ile değerlendirildiğinde 5 olgu skor 1, 56 olgu skor 2 ve 12 olgu skor 3 olarak belirlendi. İn situ hibridizasyon yöntemi görüntü analiz sistemi ile yeniden değerlendirildiğinde 6 olgu manuel değerlendirmeden farklı sonuçlanmıştır.

Sonuç: HER2/neu immünhistokimya sonuçlarının manuel olarak ve görüntü analiz sistemi kullanılarak değerlendirilmesi arasındaki uyum oranı %76'dır; in situ hibridizasyon sonuçlarının manuel ve görüntü analiz sistemi ile değerlendirilmesi arasındaki uyum oranı ise %91 olarak tespit edilmiş olup istatistiksel olarak anlamlıdır (k= 0.832 ve p<0.001).

Anahtar Sözcükler: İmmünhistokimya, İn situ hibridizasyon, Bilgisayar destekli görüntü analizi, Meme kanseri, HER2/neu

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INTRODUCTION

HER2/neu gene amplification is found in 15-25% of invasive breast carcinomas (1). These cases are the most suitable candidates for treatment with trastuzumab that has a high cost and known cardiotoxic side effects. There is no standard approach to the method to use when determining HER2/neu status in breast carcinoma patients. The oncoprotein amount may be measured using immunohistochemistry, elisa or western blot; the gene amplification amount by southern blot, in situ hybridization methods or PCR; and the m-RNA level by northern blot (2).

Immunohistochemistry (IHC) is a widely used and relatively inexpensive test. However, the subjectiveness of the evaluation process and the variability between observers make it difficult to standardize the method. The fixation and maintenance conditions of the samples also need to be optimized to obtain standard results.

In situ hybridization methods are based on the use of DNA probes to visualize and detect the copy number of the HER2/neu gene and chromosome 17 (Kr-17, CHR 17) in various ways using DNA probes. DNA probes are marked with fluorescence for fluorescent in situ hybridization (FISH), and a chromogen that can be visualized with the light microscope for chromogenic in situ hybridization (CISH) (2). DNA probes marked with silver are used in the silver-enhanced in situ hybridization (SISH) method. The HER2/neu gene copy number and Kr-17 copy number are determined separately in invasive tumoral cells and the ratio calculated. The sections are evaluated with the light microscope for cases investigated with SISH and the signals can be archived, similar to CISH and IHC, as they do not fade. SISH is a quantitative method like FISH and CISH and there is a high degree of concordance between evaluations in these methods. SISH also fits the recommendation of ASCO/CAP guidelines that the method should be 95% consistent with FISH (3).

Pathologist-assisted computer-mediated visual image analysis systems developed in recent years aim to eliminate the subjectiveness derived from manual evaluation and also make tele-consultation possible. Any field on the preparation can be examined in detail, making it easier to correctly evaluate tumor morphology and various tumor markers (4-7).

MATERIAL and METHOD

We evaluated the ErbB2 status of primary and metastatic breast tumors manually using the SISH method and ICH method in a total of 73 cases consisting of 55 modified radical mastectomies, 11 excisional biopsy material, 5 incisional biopsy material and 2 metastatic lymph node excisions.

We then re-evaluated the ICH and SISH results of these cases using the Ventana Image Analysis System (VIAS, Ventana Medical Systems, AZ, USA). We compared the degree of conformance between manual and computer-mediated visual analysis system for both methods (ICH and SISH).

Cases where the primary tumors were evaluated at our department were fixated for 6-12 hours in 10% buffered neutral formaline and embedded in paraffin blocks following routine procedures.

Afterwards, sections 5 μm thick were obtained and stained routinely with hematoxylin-eosin and immunohistochemically for HER2/neu.

HER2/neu (CB11, Ventana&Pathway) scoring was performed by evaluating membrane staining, using the ASCO/CAP (American Society of Clinical Oncology/College of American Pathologists) 2007 recommendations (9). Accordingly;

- Negative IHC staining for the HER2/neu protein, 0 or 1+: No staining or weak, incomplete membranous staining in a certain percentage of tumor cells.
- Significant IHC staining for the HER2/neu protein, 2+: Weak or non-homogenous complete membranous staining in at least 10% of the tumor cells.
- Positive IHC staining for the HER2/neu protein, 3+s: Homogenous, dense complete membranous staining in more than 30% of the invasive tumoral cells.

SISH staining was also performed, after sections 4 μm thick prepared from the best block were obtained onto slides with adhesive to study and stained with the BenchMark automatic preparation stainer (Ventana Medical Systems, AZ, USA). The SISH protocol consisted of deparaffinization, citrate addition, incubation with ISH protease, addition of the HER2/neu DNA or Kr-17 probe and incubation for hybridization, incubation with Solver C, using hematoxylin as a counterstain and incubation with bluing following counterstaining. The protease duration and incubation duration with DNA probes was optimized for each material to protect tissue morphology and make signals visible.

SISH results were evaluated according to the producer's guidelines on the light microscope (x20, x40 lens) with the the semiquantitative method (method 1) or the quantitative method (method 2 or method 2a). The HER2/neu and Kr-17 slide adequacy was determined before using this method.

The mean HER2/neu and Kr-17 signal number was determined semiquantitatively on method-1 and the ratio determined. The ratio was evaluated as follows:

1. HER/Chr17 < 1.4: Negative for HER2/neu gene amplification.
2. 1.4 ≤ HER/Chr17 ≤ 4: Borderline for HER2/neu gene amplification
3. HER/Chr17 > 4: Positive for HER2/neu gene amplification.

Method 2 was used when the results were borderline with method 1. Method 2 is a quantitative method. Once the adequacy of the slide was confirmed, HER2/neu signals were counted in 20 cells in a suitable target area within the invasive tumoral area followed by counting the Kr-17 signals in 20 cells and the ratio of the HER2/neu signals to Kr-17 signals was calculated. The assumption was that gene amplification was not present if this ratio was less than 1.8 while it was present if higher than 2.2. Method 2a was used if the ratio was ≥1.8 and ≤2.2 and the HER2/neu and Kr-17 signal was counted in another 20 cells in a suitable adjacent area in addition to the 20 cells in method 2 (a total of 40 cells). Similarly, gene amplification is not present if this ratio is less than 1.8 and present if it is more than 2.2. Cases where the the ratio was ≥1.8 and ≤2.2 were accepted as borderline and controversial.

The SISH results and ICH results for all these cases obtained by manual evaluation and the required number of different microscopic images obtained taking the same fields as the basis were again evaluated with the VIAS.

Statistical Analysis: The significance of the conformance between the VIAS and manual evaluation of SISH results and the VIAS SISH and IHC results were determined using the Kappa coefficient. A $p<0.05$ meant that the results were significant.

RESULTS

When IHC was used as the method to determine the HER2/neu status of the cases, all the 73 cases were evaluated as score 2 on manual evaluation. The same cases were evaluated as score 1 in 5 cases, score 2 in 56 cases and score 3 in 12 cases on VIAS. When the SISH method was used, one case that was borderline on manual examination was negative on VIAS while 2 positive cases and 2 negative cases on manual testing were borderline on VIAS and one positive case on manual testing was negative on VIAS. Other evaluations were consistent (Figure 1-3).

There was no statistically significant conformance between IHC results evaluated by VIAS and SISH results evaluated by VIAS (k= 0.040 and p= 0.263) (Table I). The rate of consistency between the VIAS evaluation and manual evaluation of SISH results was 91% and these evaluation results were consistent in a statistically significant manner (k= 0.832 and p<0.001) (Table II)

The HER2/neu score was 2 in all cases when the IHC was evaluated manually and we were therefore unable to

Table I: Cross-table of IHC and SISH results evaluated by VIAS

IHC VIAS	SISH VIAS			
	Negative	Borderline	Positive	Total
Score 1	5 (6,8%)	-	-	5 (6.8%)
Score 2	33 (45.3%)	5 (6.8%)	18 (24,6%)	56 (76.7%)
Score 3	9 (12.3%)	-	3 (4,1%)	12 (16.4%)
Total	47 (64.4%)	5 (6.8%)	21 (28.8%)	73 (100.0%)

Table II: Cross-table of VIAS and manual evaluation of SISH results

SISH VIAS	SISH Manual			
	Negative	Borderline	Positive	Total
Negative	45 (61.6%)	1 (%1,4)	1 (%1,4)	47 (%64,4)
Borderline	2 (2.7%)	1 (%1,4)	2 (%2,7)	5 (%6,8)
Positive	-	-	21 (%28,8)	21 (%28,8)
Total	47 (64.3%)	2 (%2,8)	24 (%32,9)	73 (%100,0)

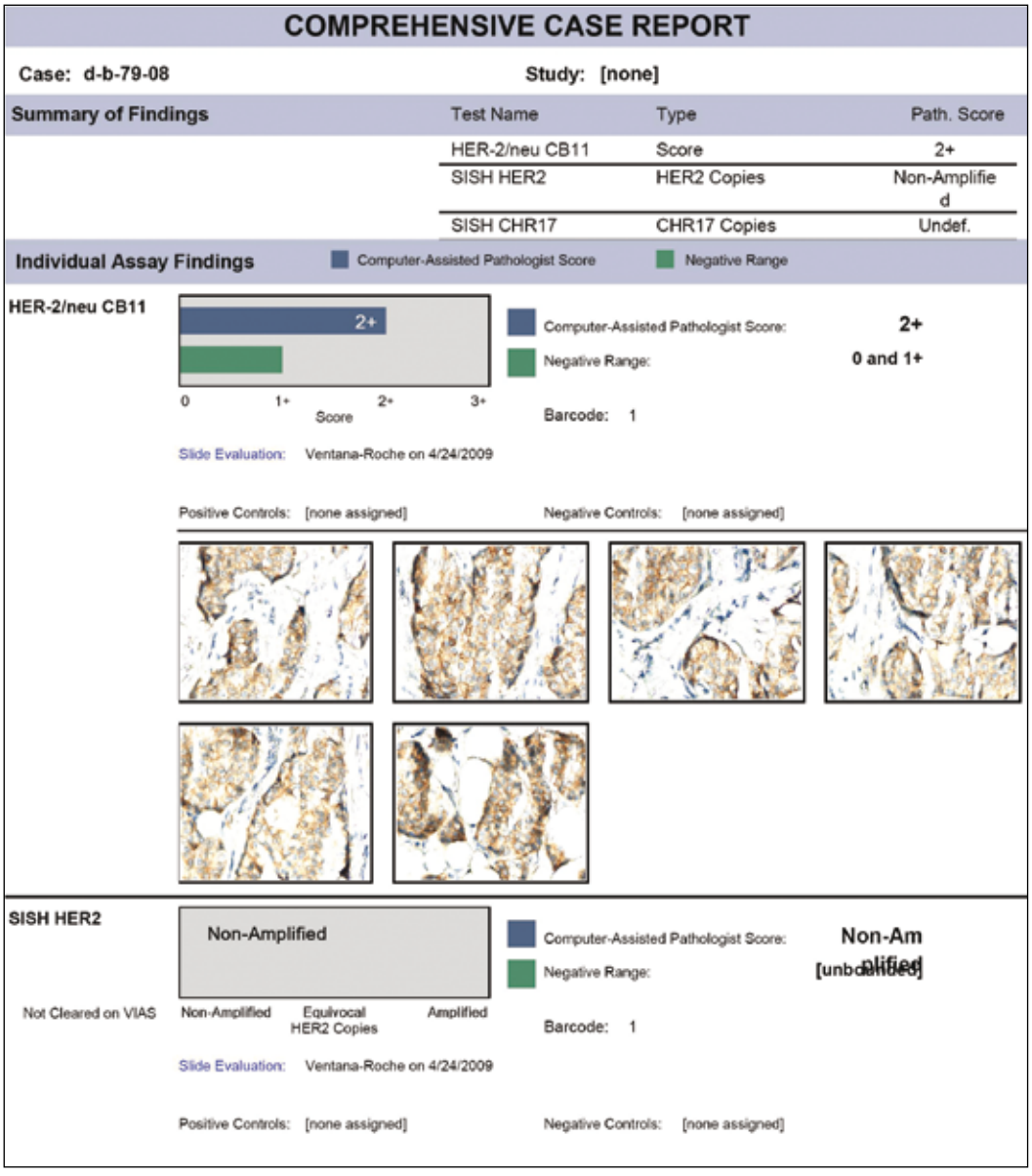

Figure 1: VIAS photographs from various areas of the case that was immunohistochemically evaluated as HER2/neu score 2.

evaluate the statistical consistency rate between the manual and VIAS IHC evaluation and similarly manual IHC and SISH evaluation. The consistency rate between manual HER2/neu IHC evaluation and VIAS evaluation results was 76%.

DISCUSSION

Final ASCO/CAP recommendations state that the test to be used to determine HER2/neu status in breast carcinoma should be more than 95% consistent with other current methods (1). Despite the standardization efforts, inconsistencies between different evaluators continue with IHC when used to determine the HER2/neu status. A study on the variability of IHC evaluation showed manual scoring differences varying between 54 and 85% among 10 different evaluations (6). Some reports state that the correct determination of biomarkers using automated computer-mediated methods is effective in determining the clinical character of the tumor and those cases that will respond to trastuzumab (8).

We were unable to perform a statistical analysis of the manual and VIAS-assisted HER2/neu IHC result evaluations as all manual results were score 2. Similarly, no statistical analysis was performed for the consistency between manual and VIAS IHC evaluation. Studies with more heterogenous groups are needed for such comparisons.

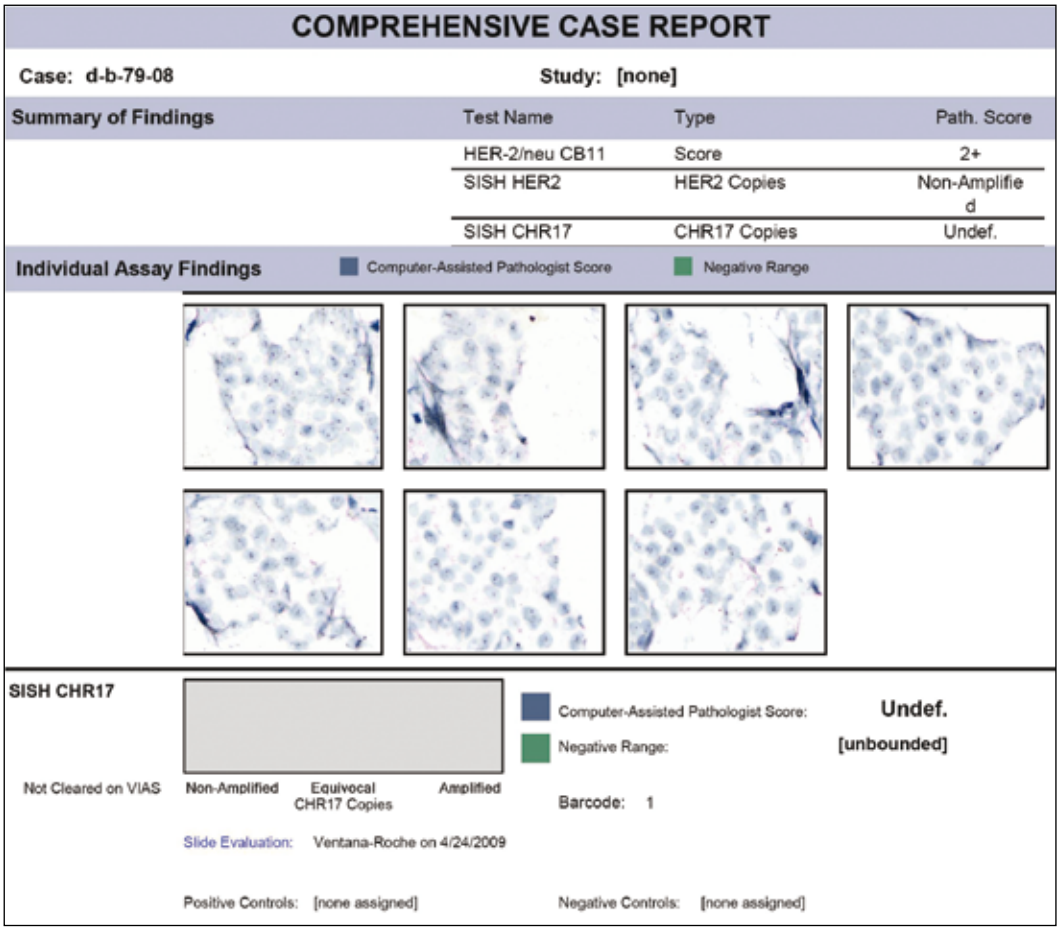

Figure 2: VIAS evaluation of the absence of HER-2 gene amplification by SISH in the same case. There is staining with a normal pattern as 1-2 spots representing HER-2 in the tumor cells.

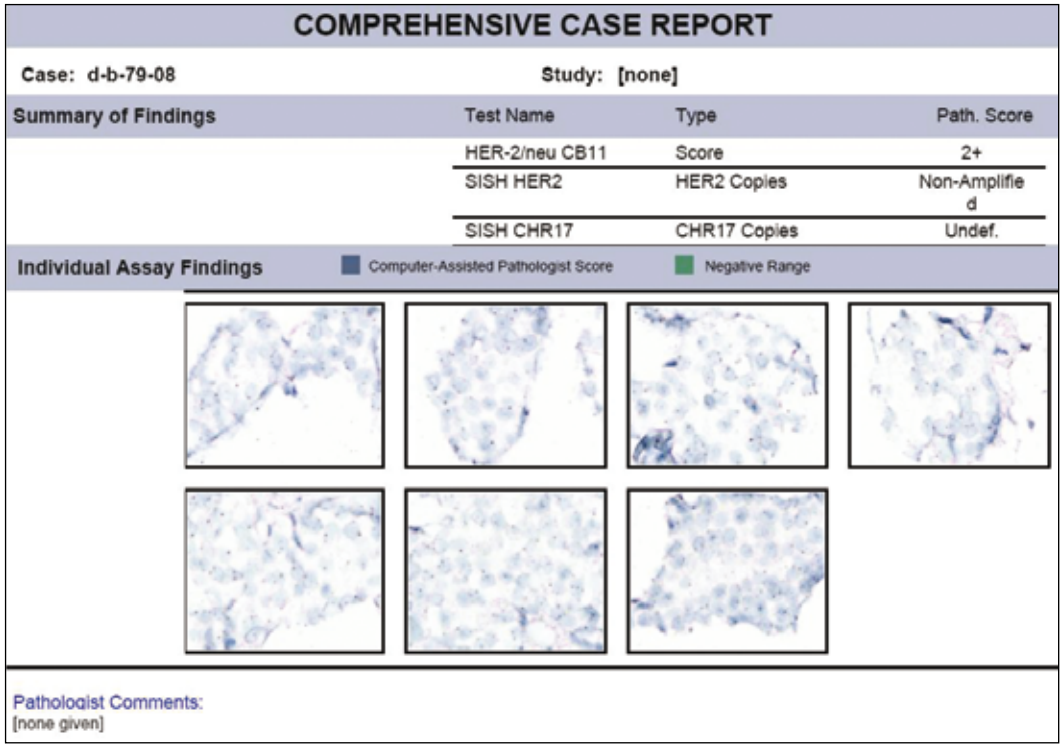

Figure 3: Kr-17 SISH preparations of the same case show staining with a normal pattern as 1-2 black spots in tumor cells. However, these have not been counted by the device and the cells where Kr-17 spots will be counted have been selected by the pathologist.

VIAS has been found to successfully select invasive tumoral areas in all cases. However, the system also makes it possible for the pathologist to exclude any areas if there are any suspicions about a particular area. Some authors see computer-mediated systems as a method that will increase the success of IHC evaluation (5,7,9). It has also been reported that inter-observer differences in evaluation due to tumor heterogenousness can be eliminated with these methods (7). However, the disadvantage of visual image analysis systems is their high cost which precludes daily practical use at all sites (5).

The consistency between manual and VIAS evaluation of SISH results was statistically significant, possibly due to SISH being an objective evaluation method. There was also only 1 case that was different as regards positivity/negativity between manual and computer-mediated evaluation of SISH results. All other results were borderline cases. This supports the notion that both are current methods for HER2/neu evaluation.

There is no statistically significant consistency between the VIAS-evaluated SISH results and IHC results. This is probably due to the difficulty in standardizing tissue follow-up and fixation that can lead to IHC staining differences.

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