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Original article

Utility of cytopathological specimens and an automated image analysis for the evaluation of HER2 status and intratumor heterogeneity in breast carcinoma

Koji Arihiro (MD, PhD)*, Miyo Oda (CT, PhD), Katsunari Ogawa (CT), Yoshie Kaneko (CT), Tomomi Shimizu, Yuna Tanaka (CT), Yukari Marubashi (CT), Katsunari Ishida (CT), Chikako Takai (CT), Chie Taoka (CT), Shuji Kimura (MD, PhD), Noriyuki Shiroma (MD)

Department of Anatomical Pathology, Hiroshima University Hospital, Japan

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ABSTRACT

Objectives: Although updated HER2 testing guidelines have been improved by a collaboration between the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) in 2013, HER2 evaluation is still problematic because of issues involving CEP17 polysomy, heterogeneity, and HER2 score 2+ cases. The aim of this retrospective study was to evaluate the relationship between HER2 gene heterogeneity, or so called CEP17 polysomy, using breast carcinoma cells sampled by scraping and the IHC score graded by automated image analysis using whole slide image.
Material and methods: We randomly selected 23 breast carcinoma cases with a HER2 score 0, 24 cases with a HER2 score 1+, 24 cases with HER2 score 2+, and 23 cases with HER2 score 3+ from the records of patients with breast cancer at Hiroshima University Hospital. We compared the results of fluorescent in situ hybridization (FISH) using formalin-fixed, paraffin-embedded (FFPE) tissues and cytological samples and compared the HER2 score calculated using an automated image analysis using wholly scanned slide images and visual counting.
Results: We successfully performed the FISH assay in 78 of 94 cases (83%) using FFPE tissues and in all 94 (100%) cases using cytological samples. Frequency of both HER2 amplification and CEP17 polysomy was higher when cytological samples were used than when FFPE tissue was used. Frequency of HER2 heterogeneity using cytological samples was higher that than using FFPE tissue, except for the IHC score 3+ cases.
Conclusions: When assessment of HER2 status based on FISH using FFPE tissue cannot be accomplished, FISH using cytological samples should be considered. When intensity of HER2 is heterogeneous in the tumor tissue, particularly in cases regarded as score 2+, they should be evaluated by automated image analysis using the whole slide image.

1. Introduction

Recently, a refined assessment of HER2 in invasive breast carcinoma has become necessary to select therapeutic agents according to the recommendations and guidelines for early or recurrent breast cancer proposed by a collaboration between the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) in 2013 [1,2]. The guidelines proposed three categories for cases with a HER2/CEP17 ratio <2.0: in situ hybridization (ISH)-positive, ISH-equivocal, and ISH-negative. In particular, ISH positive is defined as average HER2 copy number ≧6.0 signals per cell, irrespective of HER2/CEP17 ratio <2.0. This suggests a potential responsiveness to trasutuzumab-containing therapies in cases showing an elevated CEP17 count; however, the exact cut-off of CEP17 count between "trasutuzumab-containing therapies responsive" and "trasutuzumab-containing therapies response uncertain" has not yet been determined [3,4]. In addition, some problems are still to be resolved in the actual clinical and pathological diagnosis, such as CEP17 polysomy, HER2 heterogeneity, and accuracy for score 2+.

* Corresponding author at: Department of Anatomical Pathology, Hiroshima University Hospital, 1-2-3 Kasumi, Minami-ku, Hiroshima, 734-8551, Japan.
E-mail address: arihiro@hiroshima-u.ac.jp (K. Arihiro).

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Intratumoral genetic heterogeneity of HER2 is defined by the presence of >5% but <50% of invasive carcinoma cells showing a HER2/CEP17 ratio >2.2 according to a CAP Expert Panel in 2009 [5]. Based on previous reports, the frequency of HER2 genetic heterogeneity is reported to be 11%–40% [6–12]. In addition, two types of phenomena about HER2 genetic heterogeneity have been described: a clustered form, characterized by two different clones of cancer cells showing HER2 amplification or no amplification within a lesion, and a mosaic form, characterized by a mixture of HER2-amplified cells and non-amplified cells [3,4].

In terms of the clinicopathological impact of heterogeneity of HER2 status in breast carcinoma cells, although the percentage of breast carcinoma cells with a HER2/CEP17 ratio ≧2.2 has not been reported to be useful for identifying cases with heterogeneous amplification or poor outcomes [6], genetic heterogeneity is reported to be significantly associated with CEP17 polysomy, a higher histological grade, genomic instability [7], and poorer disease free survival [10]. From a therapeutic viewpoint, the response of some "HER2-negative" cases to trastuzumab suggests that genetically heterogeneous breast cancer includes a significant population of HER2-amplified breast carcinoma cells, and therefore is sensitive to anti-HER2 therapy [7,8]. However, the question of whether or not genetically heterogeneous breast cancer responds to anti-HER2 therapy remains to be answered.

On the other hand, heterogeneous overexpression of HER2 protein within a lesion has also been observed [13]. This has led to a controversy about HER2 status in breast cancer cases scored as 2+ using immunohistochemistry (IHC). In particular, the cases composed of <10% of strongly HER2-positive invasive carcinoma cells come into question because of the difficulty of interpreting IHC [14–19]. Therefore, to provide a standardized semi-quantitative measurement of HER2 protein in IHC specimens, automated image analysis technology using digital image capture involving various software systems, such as Aperio Scanscope and Automated cellular imaging system (ACIS) III [20,21], Definiens TissueStudio™ [22], Slidepath Tissue Image Analysis (IA) system [23,24], Aperio Genie Classifier [25], and HER2-CONNECT™, have been developed [26]. Although it is emphasized that computerized image analysis has improved quantification, reproducibility, and inter-observer variability for the evaluation of HER2 breast cancer, a validation study of the image analysis approach has not yet been performed in the context of HER2 heterogeneity.

The aim of this retrospective study was to evaluate the relationship between HER2 genetic heterogeneity, or so called CEP17 polysomy, and the IHC score determined using breast carcinoma cells, sampled by scraping, which were graded based on automated image analysis using the whole slide image.

2. Materials and methods

2.1. Samples

Since 2004, cytological samples have been scraped and collected from primary breast cancer patients, which have been treated as previously described for FISH analysis and kept at −80 °C until use [27]. For cases paired with cytology samples from formalin-fixed, paraffin-embedded (FFPE) tissues, five 4-μm-thin sections were serially cut and mounted on precoated slides. An H&E-stained slide of primary tumor from each case was reviewed, and the component of invasive carcinoma in the primary tumor was confirmed to be the main lesion in all cases. The histological type of each tumor was invasive carcinoma, not otherwise specified.

2.2. IHC assay for HER2 expression

To perform IHC by the DAKO system using DAKO Autostainer™ (Glosrup, Sweden) and anti-HER2 pAb, HercepTest II (DAKO) was used. For antigen retrieval, the sections were manually immersed in Target retrieval solution, high pH (DAKO), and heated in a water bath at 95 °C–99 °C for 40 min. Endogenous peroxidase activity was quenched by immersion in 3% H_2O_2 for 5 min. The tissue sections were incubated with primary antibody for 30 min at room temperature. Immunoperoxidase staining was performed using the LSAB system, ENVISION+ kit/HRP, according to the manufacturer's instructions (DAKO, Corp.), and sections were counterstained with hematoxylin. Tumors were scored according to the ASCO/CAP HER2 testing guideline update [1,2].

2.3. FISH assay for HER2 and CEP17

FISH was performed using a PathVysion HER2 DNA probe kit (Abbott Molecular, Des Plaines, Ill) on FFPE tissue and cytological samples according to the manufacturer's instructions.

2.4. Slide scanning and computer-assisted digital analysis

Stained slides were scanned using a NanoZoomer (Hamamatsu Photonics, Hamamatsu, Japan). Visualization and automated cell counts were performed using the image analysis software, Tissue Studio version 1.1 (Definiens AG, Munich, Germany), and visual evaluation of the percentage of positive cells was performed for the whole slide. If contamination by non-cancerous tissue or an intraductal component of the carcinoma was present, the pathologist could manually exclude it. Despite area selection, a certain minimum contamination of host cells is inevitable when stromal, lymphoid, and normal cells are intimately associated with tumor cells. To distinguish non-carcinoma cell elements from the non-immunostained carcinoma cell nuclei on the digitized image, nuclei with small areas (<32 μm^2 gross area, which was determined by the mean nuclear area of 50 infiltrating lymphocytes, 31.7 μm^2 and by the mean nuclear area of 50 normal ductal cells, 31.1 μm^2) and spindle features (>0.5 oval rate) were regarded as lymphocyte, normal ductal cell, and stromal cell nuclei, respectively, and were eliminated.

2.5. Evaluation of FISH and IHC

We used scoring systems based on the American Society of Clinical Oncology/College of American Pathologists recommended guidelines to evaluate the FISH findings [1,2]. The CEP17 polysomy was defined as a mean of CEP17 signal number >3 per 20 nuclei based on FISH using FFPE tissues and cytological samples. The heterogeneity of HER2 was defined by the presence of >5% but <50% of invasive carcinoma cells showing a HER2/CEP17 ratio >2.2 based on FISH using FFPE tissues and cytological samples according to a CAP Expert Panel in 2009 [28]. The heterogeneity of HER2 was defined by the presence of >0% but <10% of invasive carcinoma cells showing a strong intensity of HER2 in IHC, as evaluated by the automated image analysis. All study specimens were scored by two different examiners (K.A. and M.O.) who were blinded to the patient's characteristics.

2.6. Statistical analyses

The relationships between the categorical variables were analyzed using a chi-square test and Fisher's exact probability test. The case-by-case comparison of the HER2/CEP17 ratio between the FFPE tissues and cytological samples was analyzed using a Wilcoxon signed rank test. Associations between continuous variables were analyzed using nonparametric Spearman rank correlation coefficients.