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Original Article

Evaluation of FISH image analysis system on assessing HER2 amplification in breast carcinoma cases

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Received 28 December 2006; received in revised form 30 May 2007; accepted 12 July 2007

Abstract

HER2-positive breast cancer is characterized by aggressive growth and poor prognosis. Women with metastatic breast cancer with over-expression of HER2 protein or excessive presence of HER2 gene copies are potential candidates for Herceptin (Trastuzumab) targeted treatment that binds to HER2 receptors on tumor cells and inhibits tumor cell growth. Fluorescence in situ hybridization (FISH) is one of the most widely used methods to determine HER2 status. Typically, evaluation of FISH images involves manual counting of FISH signals in multiple images, a time consuming and error prone procedure. Recently, we developed novel software for the automated evaluation of FISH images and, in this study, we present the first testing of this software on images from two separate research clinics. To our knowledge, this is the first concurrent evaluation of any FISH image analysis software in two different clinics. The evaluation shows that the developed FISH image analysis software can accelerate evaluation of HER2 status in most breast cancer cases.

Keywords: HER2; Fluorescence in situ hybridization (FISH); Automated image analysis

Introduction

HER2 gene amplification is prognostically and therapeutically significant for patients with breast cancer,[1] occurring in approximately 20–30% of metastatic breast carcinomas.[2] Accurate determination of HER2 status is important in the management of patients with breast cancer, especially in determining their eligibility for trastuzumab therapy.[3] Over-expression of the protein product of HER2 gene is usually a consequence of gene amplification, in which multiple copies of the gene appear throughout the genome. It is thus possible to determine HER2 status by analyzing the gene copies or the protein expression.

A variety of methods are available for the detection of HER2 status; however, for clinical routine and research, the most widely applied techniques are immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH). Both techniques permit the study of small amounts of formalin-fixed, paraffin-embedded tissue and the interpretation of the findings on a cell-by-cell basis. IHC uses specific antibodies to stain proteins (products) in situ, which allows the identification of many cell types that

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doi:10.1016/j.breast.2007.07.041

Please cite this article as: Theodosiou Z, et al. Evaluation of FISH image analysis system on assessing HER2 amplification in breast carcinoma cases. *The Breast* (2007), doi:10.1016/j.breast.2007.07.041

could be visualized by classical microscopy. Advantages of IHC testing include its wide availability, relatively low cost, easy preservation of stained slides and use of a routine light microscope. Disadvantages of IHC include the impact of pre-analytic issues including storage, duration and nature of system control samples, and most importantly, the difficulties in applying a subjective slide scoring system.[4]

FISH imaging allows selective staining of various DNA sequences and thereby the detection, analysis and quantification of specific numerical and structural abnormalities within nuclei. FISH has the advantages of a more objective scoring system and the presence of a built-in internal control consisting of the two HER2 gene signals present in all non-neoplastic cells in the specimen. Fluorescence in situ hybridization is a direct in situ technique that is relatively rapid and sensitive. No cell culture is needed in order to apply this method and results are easier to interpret than karyotype. Disadvantages of FISH testing include the high cost of each test, long time needed for slide scoring, high acquisition cost for a fluorescence microscope and occasional difficulty in identifying the invasive tumor cells.[4]

Manual evaluation of FISH images is a time-consuming and error-prone process involving manual counting of FISH signals over a tissue slide. In order to address these issues, many studies have focused on automated evaluation of FISH images using image processing methods.[5–9]. In addition, several methods are commercially available for the determination of the HER2 receptor status in breast cancer. In a recent study, Klijanienko et al.[10] utilized the Discovery automated image analyzer (Becton Dickinson, Leiden, Netherlands) to quantify the level of amplification in highly amplified tumors when the amounts of signal were not countable by visual analysis. Ellis et al.[11] performed image analysis on breast cancer samples using the HER2 digital scoring application of the Metastasis Detection System (MDS^{TM}; Applied Imaging, San Jose, California, USA). Their results showed that FISH scoring using the MDS system was highly consistent and less complicated than manual scoring. However, the system was slower than the manual scoring, on non-amplified FISH cases, potentially rendering it impractical for clinical use.

In practice, analysis of FISH images is currently performed in a semi-automated way, with the aid of image processing software, which can display the different color channels of a FISH image.[12] A study by Klijanienko et al.[10] has shown strong correlation of detection results using visual-only and semi-automated methods for evaluating the status of HER2 in breast carcinomas samples. However, the counting of dots in a semi-automatic manner still remains impractical for a pathologist, since it requires user intervention for excluding poorly segmented, overlapping, clustered or non-epithelial cells,[10] while not reducing the time requirements of the process.

Here, we have developed novel automated system that aims to address these issues. The algorithmic basis of the system is described in Raimondo et al.[12] The system was used in two parallel evaluation studies at two different institutions, the University of Pisa and the Aristotle University of Thessaloniki, thus yielding the first, to our knowledge, evaluation of its kind.

Materials and methods

Formalin fixed–paraffin embedded tissue blocks from 100 cases were retrieved from the archives of Pathology Department of the Aristotle University Medical School and the Division of Surgical Molecular and Ultrastructural Pathology of university of Pisa.

FISH slide preparation by University of Pisa

FISH was performed using fluorescent-labelled DNA probes obtained from Vysis. Five-micrometer think paraffin sections were deparaffinated, deydratated and incubated in 2 × SSC a 75% for 15 min and digested in pepsin solution (4 μg/ml in 0.9 NaCl, pH 1.5) for 15 min at 37 °C, rinsed in 2 × SSC at room temperature for 5 min and air dried. Dual probe hybridization was performed using a spectrum-green labelled probe for the centromere region of chromosome 17 (CEP-17) and a spectrum orange/red-labelled probe for HER2. Probes and target DNA were denaturated simultaneously in an 85 °C oven for 1 min and hybridization was performed by incubation at 37 °C for 16−18 h. Post-hybridation washes were performed using wash buffer (2 × SSC/0.3%, NP-40) a 72 °C for 2 min. Nuclei were counterstained with DAPI and antifade compound *p*-phenyldiamine. The number of HER2 signal and CEP-17 signals were counted in 60 non-overlapping nuclei and an overall mean HER2 to CEP-17 ratio was calculated. Tumors with ratios greater than or equal to 2.0 were defined as having HER2 amplification. An example of FISH image provided by the University of Pisa is shown in Fig. 1(a).

FISH slide preparation by Aristotle University of Thessaloniki

The copy number of HER2 gene locus at 17q11.2–q12 and alpha satellite DNA located at band region

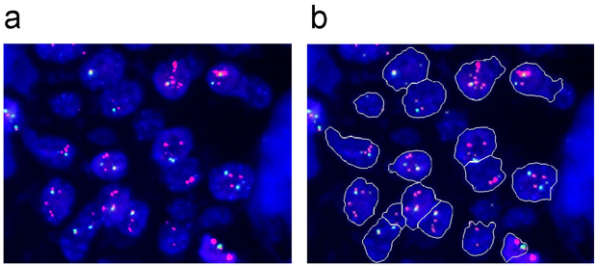

Fig. 1. Automated spot detection and nuclei segmentation for a UPisa data set sample image. (a) The input FISH image. (b) The processed image after image analysis. Segmented nuclei are shown in outline and detected FISH signals as cross marks.

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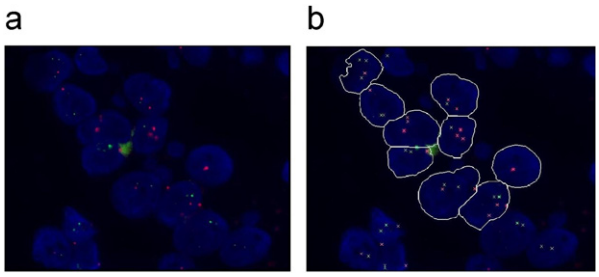

Fig. 2. Automated spot detection and nuclei segmentation for an AUTH data set sample image. (a) The input FISH image. (b) The processed image after image analysis. Segmented nuclei are shown in outline and detected FISH signals as cross marks.

17p11.1–q11.1 (CEP17) was estimated by FISH in interphase cells on paraffin TMA sections(3.5 μm), directly labelled with the PathVysion™ HER2 DNA probe (Vysis) according to the manufacturer's instructions. Briefly, the sections were deparaffinized by overnight heating at 60 °C and by two xylene washes for 5 min each time, followed by dehydration in 100% ethanol for 5 min twice. The slides were air dried and immersed in pretreatment solution (NaSCN) at 80 °C for 30 min. Proteolysis of neoplastic cells was performed by immersing the slides in protease solution at 37 °C for 12 min. Denaturation of tissue sections mounted on the slides was performed by a solution of formamide in 70%, pH 7.5, at 72 °C for 5 min. Hybridization was carried out by adding to the tissue sections 10 μL of LSI HER2/CEP17 DNA probe for overnight incubation at 37 °C in a moist chamber. Next day the slides were washed with post-hybridization buffer (2 × SSC and 0.3% NP-40) at 72 °C for 5 min. Hybridization signals were enumerated in a Zeiss microscope (Axioskop 2 plus HBO 100) equipped with a high quality ×100 oil immersion objective, an appropriate filters set (EX BP360/51 for DAPI, EX BP485/17 for FITC/spectrum green, EX BP560/18 for rhodamine/spectrum orange/red) and a computerized imaging system. Sixty non-overlapping nuclei were selected randomly and scored for each tumor specimen. HER2 probe is labelled in spectrum orange/red and the CEP 17 probe in spectrum green. HER2 gene copies status was estimated as the ratio between the total number of HER2 gene signals and the number of chromosome 17 centromere signals. Amplification was considered if the ratio of HER2/CEP-17 was >2.0. Images were captured with a computer-controlled digital camera and processed with a software system (FISH Imager™ METASYSTEMS). An example of FISH image provided by the Aristotle University of Thessaloniki is shown in Fig. 2(a).

FISH image analysis system

The FISH image analysis system was designed to accelerate FISH image evaluation while increasing evaluation accuracy. It was developed as a module and integrated into the Volumetric Image Processing, Analysis and Visualization software package, EIKONA3D (Alpha Tec Ltd, http://www.alphatecltd.com). The system boasts a graphical user interface (GUI), making it very practical and simple to use. FISH image evaluation is performed via the algorithm described in Raimondo et al.[12]

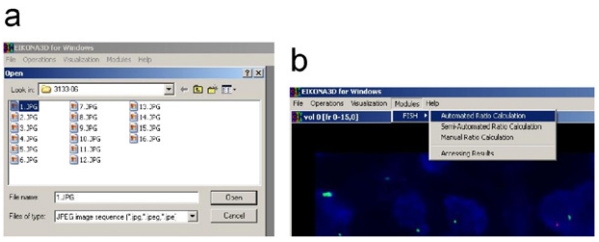

Fig. 3. FISH system graphical user interface (GUI). (a) Image loading onto the buffer. (b) Automated, semi-automated and manual mode selection.

Software operation is user-friendly and intuitive. As shown in Fig. 3(a), the images, which can be of a number of supported formats, are initially loaded onto an input buffer for processing. Next, as shown in Fig. 3(b), the user selects one of three available modes for FISH image analysis: automated, semi-automated and manual. In the automated mode, the system automatically detects FISH signals, i.e. red/orange and green spots, and performs nuclei segmentation. This is carried out for every image of the case and results from multiple images which are combined at the end of processing. In this mode, the system calculates the average spot ratio among all valid nuclei, which are nuclei which have at least one red spot.

In the semi-automated mode, the system detects FISH signals and segments the nuclei as in the automated mode. However, the average spot ratio for each case is not calculated until the user is given the option to manually correct the spot detection and nuclei segmentation results. This capability is particularly useful in cases with very low image quality. Finally, in the manual mode, only the user is able to identify the nuclei and the locations of FISH signals. This mode can very practical act as an aid in FISH image evaluation in decision support systems.

Results

Our study consisted of a total of 100 breast cancer cases, which were classified separately by an expert and the proposed FISH image analysis system. Analysis was performed separately for the 30 cases provided by the University of Pisa and the 70 cases provided by the Aristotle University of Thessaloniki. The first group contained 30 cases collected from the division of Surgical Molecular and Ultrastructural Pathology of the University of Pisa and the second group consisted of 70 cases from the Pathology Department of the Medical School of the Aristotle University of Thessaloniki. The former group is labelled UPisa data set while the latter AUTH data set. An example of an image from the UPisa data set, before and after processing, is shown in Fig. 1(a) and (b), respectively, while an example for the AUTH data set is shown in

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Table 1
Evaluation of system performance

	UPisa			AUTH			UPisa & AUTH		
	Pos.	Neg.	All	Pos.	Neg.	All	Pos.	Neg.	All
Number of cases	7	21	28	20	50	70	27	71	98
Undetermined	1	1	2	5	0	5	6	1	7
Accuracy (%)	85.7	100	96.4	70	100	91.4	74.1	100	92.8
Accuracy w/o undetermined (%)	100	100	100	93.3	100	98.5	95.2	100	98.9

Fig. 2(a) and (b). In Figs. 1 and 2, we show the results of nuclei segmentation in outline, while the FISH signals locations are indicated with cross marks.

From the UPisa data set, 21 cases were diagnosed as negative, 7 cases as positive, while 2 cases were not classified due to bad image quality and were not available for the system. As shown in Table 1, the automated system correctly classified all negative cases, while only one out of the positive cases was classified incorrectly. The AUTH data set contained 50 negative cases and 20 positive cases. As shown in Table 1, we use two distinct methods to gauge the accuracy of our software. In the first approach we include all cases, while in the second, we exclude cases where our software produced a spot ratio in 1.8–2.2 range (Pathvysion test), which are considered as ''borderline amplified''.

In Table 1 we show that, following the first method, all negative cases were correctly classified, while six positive cases were classified incorrectly. Given these data, the overall accuracy of the system on both data sets is 92.8%, with 100% accuracy on negative cases and 74.1% on positive cases. On the UPisa data set, the overall accuracy is 96.4%, with the positive accuracy 85.7% and the negative accuracy 100%. On the AUTH data set, the overall accuracy is 91.4%, with the positive accuracy 70% and the negative accuracy 100%. However, using the second method, the accuracy of the system increases to 95.2% for positive cases and 98.9% overall. For the UPisa set, overall accuracy is 100%, while one positive and one negative case are undetermined and for the AUTH cases, overall accuracy 98.46% and accuracy for positive cases is 93.3%, while five positive cases are undetermined.

Discussion

The FISH technique is a very useful diagnostic and experimental tool, allowing estimation of various genetic alterations in a large spectrum of specimens. Is a preferable technique for detection of chromosomal breakpoints because allows the detection of widely dispersed breakpoints.[13] Furthermore, FISH is more sensitive than quantitative PCR in detection of increased EGFR gene copy numbers.[14] In this study, we have presented a parallel evaluation of a newly developed FISH image analysis system on HER2 breast carcinoma data sets provided by two different institutions, the University of Pisa, Italy and the Aristotle University of Thessaloniki, Greece. To our knowledge, this is the first large scale parallel study of its kind.

As shown in Table 1, the accuracy of our software is satisfactory, especially when labelling cases with spot ratio between 1.8 and 2.2 as undetermined. These cases constitute a small minority and can be further evaluated manually by a clinician. However, there do exist few cases where low image quality has produced erroneous classification results. These occur most often in positive cases, which is expected as low image quality negatively affects the ability to detect FISH signals. Another advantage of our system, is the significantly shorter evaluation time. The average time for manual evaluation of 60 tumor nuclei in a typical non-amplified or fully amplified case requires approximately 10 min or less time. In cases with equivocal amplification, probe signals heterogeneity, background noise or weak signals, the time of evaluation is increased. The automated evaluation as here we present, requires maximum of 5 min on an average PC, with additional 3−5 min in each case for a quick manual evaluation. Given the ever-increasing improvements in microprocessor technology, automated evaluation time is sure to decrease even further, rendering automated methods, such as ours, even more attractive.

According to the practice of pathologist experts at the Aristotle University of Thessaloniki, evaluation of the HER2 status of a case based on FISH demands at least 60 valid nuclei on which the average spot ratio is calculated. When more than 60 valid nuclei are available, 60 nuclei are selected by the clinicians according to their judgement. Our automated software does not include any procedure for selecting the 60 ''best'' nuclei and instead calculates the average ratio on all valid nuclei. As a result, the average ratios calculated by the system and the clinicians could never be the same, even if spot detection and nuclei segmentation were perfect. Therefore, one of the main challenges lies in establishing and developing automatic criteria for nuclei selection.

As a conclusion, the present study demonstrates that an automated FISH image analysis system can greatly facilitate FISH image evaluation with relatively low error. However, the system still needs improvements before widespread clinical use, since it does not perform as well

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on ambiguous or border line cases, while further algorithmic improvement of our system is required in order to increase diagnostic throughput and accuracy.

Acknowledgments

This work was supported by the EU project Biopattern: Computational Intelligence for Biopattern Analysis in Support of eHealthcare, Network of Excellence Project no. 508803.

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