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RESEARCH

Computer Aided Semi-Automated Evaluation of HER2 Immunodetection—A Robust Solution for Supporting the Accuracy of Anti HER2 Therapy

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Abstract HER2-positive breast cancers usually benefit from anti-HER2 therapy, thus, HER2 evaluation became inevitable for patient selection. HER2-negative (IHC 0, 1+) and strong positive (IHC 3+) cases can easily be interpreted with immunohistochemistry, but equivocal (IHC 2+) cases require further analysis of *HER2* gene amplification using *in situ* hybridization. Our study aimed to validate digital pathology and automated image analysis for unbiased evaluation of HER2 immunostains. We developed an image segmentation algorithm for analyzing HER2-immunostaining (4B5 clone) in tissue microarrays of breast cancers. Two pathologists assessed 309 microscopic regions of at least 100 tumor cells each—representing all HER2 positivity groups—according to international guidelines either semi-quantitatively or by using the *MembraneQuant* software. Scoring results were statistically correlated with each other and with FISH data, and almost perfect agreement was found (inter-method Cohen's kappa=0.872, Spearman-rho=0.928). When clinical relevance (scoring disagreement that may define erroneous treatment selection) was examined high agreement was found (quadratic weighted kappa=0.967). Image analysis classified cases with excellent correlation with visual evaluation, therefore, *MembraneQuant* software proved to be a reliable tool for assessing HER2 immunoreactions and supporting better targeting anti-HER2 therapy. As digital analysis of immunomorphological markers allows permanent archiving, standardization and accurate reviewing of results, it supports quality assurance initiatives in diagnostic pathology—especially of equivocal cases which are hard to interpret.

Keywords Breast cancer · Digital pathology · Validation · Image analysis · HER2 · Immunohistochemistry

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Introduction

The type 1 receptor tyrosin kinase HER2 is a member of the epidermal growth factor receptor family (EGFR1-4). *HER2* gene is found to be amplified in 15–30 % of invasive breast cancers causing reduced disease free and overall survival [1]. Targeted therapy by anti-HER2 antibodies can prevent receptor dimerization and thus block EGFR-signal transduction cascade, which together with the facilitated Antibody Dependent Cellular Cytotoxicity (ADCC) results in reduced tumor-progression and improved survival rates in HER2-positive cancers [2]. By now HER2 evaluation has become an essential part of the pathology report as a predictive marker for anti-HER2 therapy [3].

HER2 is routinely tested with immunohistochemistry (IHC) according to the ASCO/CAP guidelines using a 4-tiered classification [4]. Breast cancers with no or weak incomplete membranous HER2 immunostaining (IHC 0, 1+) are considered to be negative with no benefit from anti-HER2 therapy, while strong and complete membrane staining (IHC 3+) can be the direct indicator of a successful anti-HER2 therapy. Equivocal (IHC 2+) cases—bearing weak to moderate, but complete membranous staining—need further confirmation of HER2 gene amplification to be eligible for anti-HER2 therapy, mostly with fluorescent in situ hybridization (FISH) or SISH (silver enhanced ISH) or CISH (chromogenic ISH) techniques [5].

HER2 immunohistochemistry has been used for more than a decade, still rather high rate of inter-observer variability of interpretation has been reported [6, 7] and recent observations call attention to the heterogeneity of HER2 expression/amplification [8]. Today, the response rate for the expensive anti-HER2 therapy is still only ~50 % and the therapy may also have dangerous side effects, especially in patients with coronary heart disease [9]. According to a meta-analyses almost 20 % of anti-HER2 therapies are inadequate [1, 4, 10].

Rapidly developing digital microscopy offers powerful image analysis applications for supporting standardization, accuracy and documentation of the testing of diagnostic markers [7]. Recent international guidelines recommend the use of computer aided assessment of HER2 immunohistochemistry and FISH results for improving the consistency of the evaluation-process and potentially the efficacy of patient selection for anti-HER2 therapy [11–13].

The *Pannoramic Viewer* platform (3DHISTECH Ltd, Budapest, Hungary) offers a ready-to-use application for semi-automated scoring of immunostained signals. *MembraneQuant (MQ)* automatically detects the continuity and intensity of HER2 cell membrane reaction in each cell at the annotated area of interest. These individual values (0 to 3) can be later combined into a field score according to the thresholds set up in line with recent international guidelines.

Our study aimed to validate the *MembraneQuant's* scoring against the empirical scoring by pathologists and we found high correlation between the results gained with the two approaches. Therefore, automated image analysis of HER2 immunostaining using the *MembraneQuant* platform can be a valuable help of the accurate patient selection for anti-HER2 therapy.

Materials and Methods

Samples and Immunohistochemistry

Study was permitted by the Regional Ethics Committee of the Semmelweis University, (no. 7/2006). Formalin fixed paraffin embedded blocks of 107 females (aged 26–86 years) from the 1st Department of Pathology and Experimental Cancer Research, Semmelweis University were used to create three tissue microarrays (TMA) using *TMA Master* (3DHISTECH Ltd., Budapest, Hungary). Two hundred seven pieces of 2 mm duplicate cores were taken from 16 ductal carcinoma in situ, 126 primary invasive ductal carcinomas, 4 invasive lobular carcinomas and 61 metastases.

Slides were stained according to manufacturer's protocol on a Bond-Max™ automated staining system (Leica Microsystems GmbH, Germany), using PATHWAY® HER-2/*neu* (4B5) rabbit monoclonal antibody with DiAmino Benzidin (DAB) (Ventana Medical Systems Inc., Tucson, USA). FISH-reactions were performed on IHC + ve cases with Kreatech HER2/SE17 FISH-probes (Cat.No.KBI-10701, Kreatech, The Netherlands).

Membrane Detection Algorithm

Digitalization was performed using a *Pannoramic SCAN* (3DHISTECH, Budapest, Hungary) with a resolution of 0.2325 μm/pixel. Several image analysis processes have been published including color separation methods [14–18], which were successfully applied for IHC quantification. *Membrane Quant* analysis began with the separation of the immunoreactive cell-membranes (brown color—DAB) from non-immunoreactive elements (blue counterstain—hematoxylin) by un-mixing the original image along a vector line for color predefined channels in the RGB (red-green-blue). In this step the targeted colors were defined by their RGB values, by sampling from the tissue image finally saved to a color description file (*.ccc-file) as a loadable color profile. By applying this feature, the system can be calibrated to any selected histological IHC staining protocol and used recording system (Fig. 1).

This color deconvolution algorithm generated two different grayscale images which were separately processed in the following steps of the segmentation (Fig. 2). The membrane detection algorithm ran on the brown channel, whereas the non-immunoreactive epithelial cells (without membraneous stain) could be detected based on their cell nuclei stained by hematoxilin running on the blue channel. The processing of the cell nuclei segmentation was similar to the *NuclearQuant* application, as described previously [19].

We applied a new segmentation method where membrane sections were described as connected pixel curves of local minima of the intensity in the brown channel (DAB image). The images were segmented into adjacent spots, the border of which potentially marked the middle of the membranes lines (i.e. skeleton). After successful subtraction of membranes and nuclei, segmented masks were revealed (Fig. 2). Non-specific signals—not representing membranes—were disclosed based on their size. Average intensity of the DAB image was measured along the border of spot locations. Cells were scored

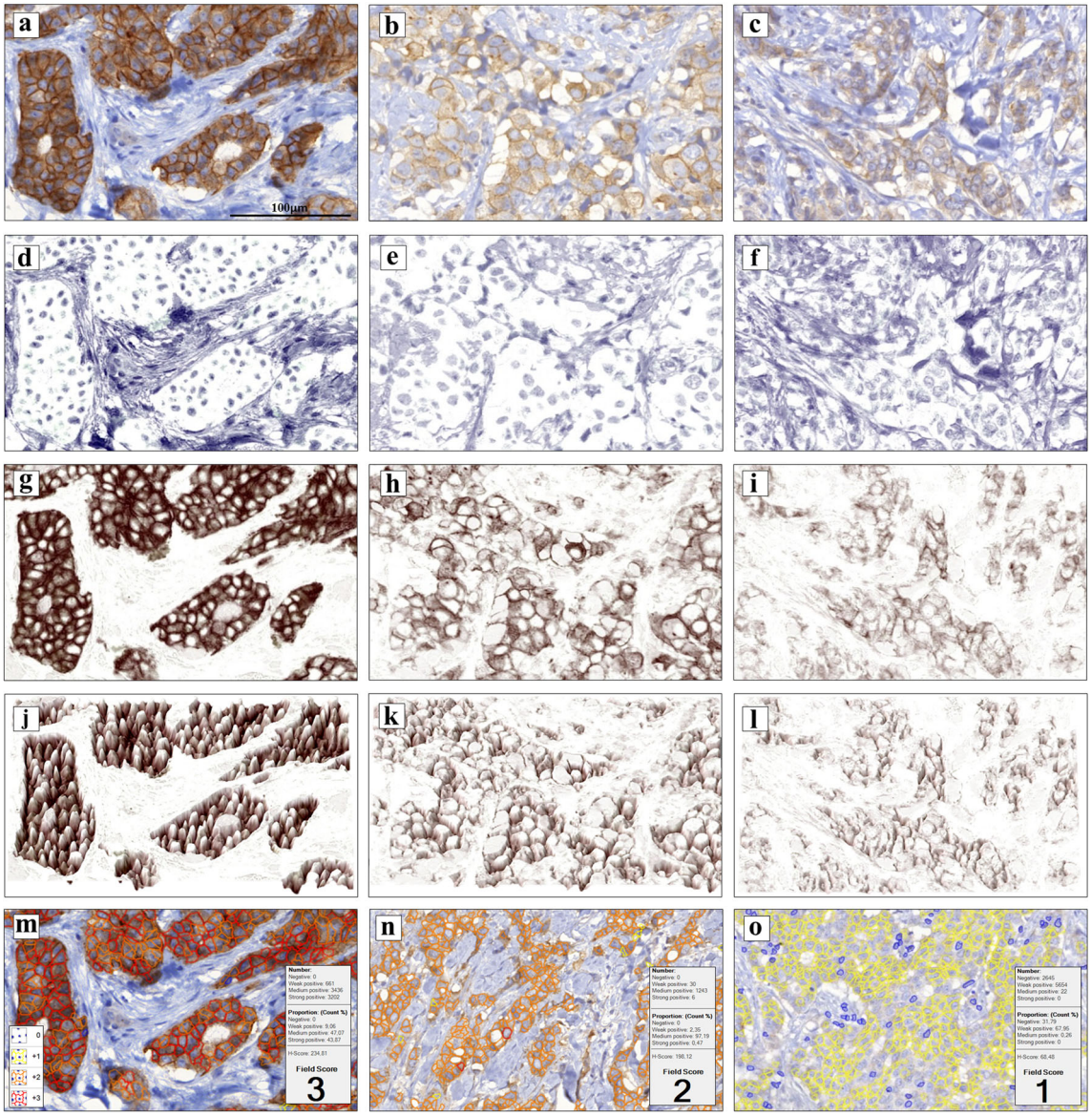

Fig. 1 Representative images of the feature outputs at different segmentation steps. The figure represents images of all positivity ranges from weak to strong positive (**a**–**c**). Two individual channels were generated using Color Deconvolution method: the immunopositive tissue elements were presented in brown (DAB-DiAminoBenzidin signal) (**g**–**i**) and the counterstain (hematoxilin) in *blue* channel (**d**–**f**). The brown channels were analyzed based on intensity (**j**–**l**). The final results are represented with outline mask and on data panels (**m**–**o**)

based on the predefined intensity thresholds corresponding to international guidelines and the classification formula. In several cases the HER2 IHC staining resulted in a non-continuous membrane stain which had a diagnostic role to distinguish equivocal HER2 IHC 2+ signals from the strong positive IHC 3+ signals. In order to manage this effect, a calibratable standard deviation setup of the intensity threshold control has been integrated into the scoring setup of the application.

Scoring of the Slides

Thirty-four TMA-cores were excluded due to unsuitable quality for an exact assessment, leaving 173 cores for analysis. Visual scoring was performed by two pathologists (T.K. and T.M.), blinded for the original HER2 scores. To enable a better inter-rater comparison and minimize the effect of intratumoral heterogeneity, a pathologist marked 1 to 6 annotations of tumorous tissue as regions of interest (ROI) on each core, which were individually scored by both pathologists and later by *MembraneQuant*. Discrepant and/or equivocal cases were re-reviewed jointly and a *Consensus Score* was provided for all 309 ROIs. The ROI-scores of the same core were summarized into an overall score that was used for the data analysis.

Scoring of HER2 immunostained and FISH slides was performed when ASCO/CAP Guidelines from 2007 [4] were valid using the cutoff value of 30 % for 3+ cases. Recently,

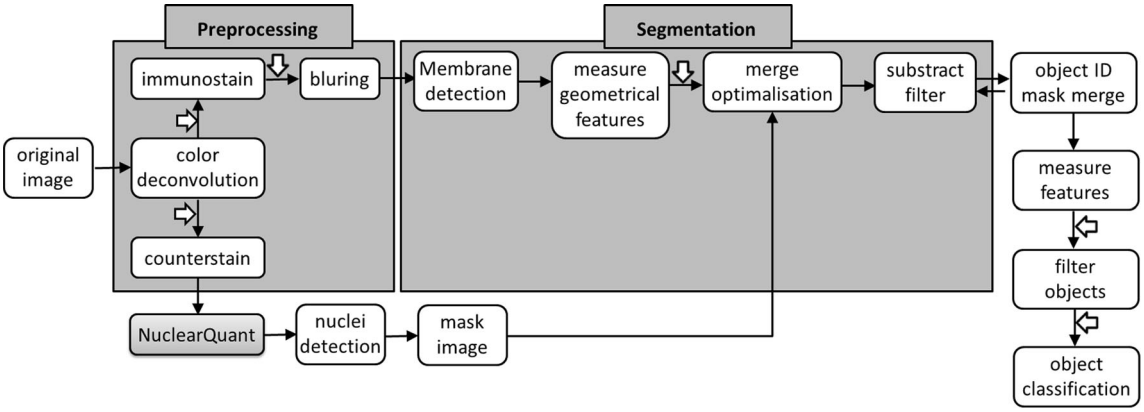

Fig. 2 Summary of algorithms applied to measure HER2 membrane immunoreactivity. In the preprocessing step a Color Deconvolution method is used to separate the immunostained (DAB-DiAminoBenzidin) signals from the non-immunoreactive (hematoxilin stained) tissue elements. The positive cells are detected based on their stained membrane particles. Negative cells can be detected based on the localization of their nuclei, thus the NuclearQuant algorithm detecting the nuclei is integrated into MembraneQuant to define the non-immunoreactive cells. Segmentation is based on the object detection algorithm and merge optimalization. *Arrows* denote the workflow steps with manual correction possibilities

a new and upgraded guideline came out in 2013 setting back the cutoff value to 10 % of tumor cells [20], which recommendation we couldn't follow retrospectively.

Statistics

Data analysis was performed using SPSS 17.0 for Windows (SPSS Inc., Chicago, USA) and MedCalc for Windows v. 11.2.1.0 (MedCalc Software, Mariakerke, Belgium). The *Consensus Score* was compared as gold standard to *MembraneQuant* with inter-method Cohen's kappa and Spearman rank-correlation coefficient. The strength of agreement was interpreted as proposed by Landis & Koch [21]. Quadratic weighted kappa was also calculated [19] by assigning different weights for more and least relevant disagreements.

Results

The inter-method kappa showed substantial to almost perfect agreement with Cohen's kappa (*MembraneQuant* vs. Path1: 0.668; *MembraneQuant* vs. Path2: 0.663 and *MembraneQuant* vs. *Consensus Score:* 0.872). The inter-method comparison with quadratic weighted kappa—testing the clinical relevance—showed also almost perfect agreements in all settings (0.905; 0.901 and 0.967, respectively), and strong/almost perfect correlation with Spearman's rho (0.861; 0.842 and 0.928, respectively) (Table 1.)

Results were re-analyzed after the removal of the metastatic cores. The assessment of the 135 carcinoma cores provided similar results with all cases: substantial and almost perfect agreements using Cohen's kappa (0.629; 0.635; 0.868, respectively), almost perfect agreement using quadratic weighted kappa (0.906; 0.901; 0.970, respecively), and strong and highly significant correlation with Spearman's rho (0, 864; 0.840; 0.937, respectively) (Table 2).

Perfect agreement was found between all scorings with the four invasive lobular carcinomas (data not shown).

During visual assessment 12 cases were scored into HER2 IHC 2+ class, which were also analyzed with *MembraneQuant* reaching an almost perfect correlation: the software application scored almost the same number of cores (11 cases) into the 2+ score group.

The immunohistochemically positive cases (HER2 2+ and 3+) were also tested with FISH and half of the IHC 2+ cases and all IHC3+ cases showed *HER2*-amplification. Altogether, 12 IHC 2+ and 23 IHC 3+ cases were tested and showed perfect agreement regarding *Consensus Score* and *MembraneQuant Score*, as shown in Table 3.

Table 1 Agreement between the different HER2-scorings for all TMA cores. (p is <0.0001 in all cases.)

Variables	Cohen's κ (95 % CI)	Quadratic weighted κ	Spearman's rho (df, 95 % CI)
MembraneQuant vs. Pathologist1	0.668 (0.575–0.761)	0.905 (0.870–0.941)	0.861 (172, 0.817–0.895)
MembraneQuant vs. Pathologist2	0.663 (0.569–0.756)	0.901 (0.864–0.938)	0.842 (172, 0.793–0.881)
MembraneQuant vs. Consensus	0.872 (0.805–0.939)	0.967 (0.947–0.986)	0.928 (172, 0.904–0.946)

Table 2 Agreement between the different HER2-scorings for primary carcinomas, when metastases are excluded. (p is <0.0001 in all cases.)

Variables	Cohen's κ (95 % CI)	Quadratic weighted κ	Spearman's rho (df, 95 % CI)
MembraneQuant vs. Pathologist1	0.635 (0.528–0.743)	0.906 (0.868–0.945)	0.864 (134, 0.815–0.902)
MembraneQuant vs. Pathologist2	0.629 (0.521–0.738)	0.901 (0.861–0.942)	0.840 (134, 0.783–0.884)
MembraneQuant vs. Consensus	0.868 (0.792–0.946)	0.970 (0.949–0.990)	0.937 (134, 0.912–0.955)

Discussion

The aggressive, HER2-positive breast cancers can benefit from targeted anti-HER2 therapy, thus accurate determination of HER2 expression/gene amplification is crucial. Patient selection can be performed by HER2 IHC and/or FISH techniques according to international guidelines. Despite the strict selection process the response rate to the expensive anti-HER2 therapy remains around 50 % [9].

This insufficient response is partly due to the complexity of growth regulation pathways, however, in about 20 % of cases inappropriate HER2 testing and/or interpretation have been blamed in the background [1, 4, 10, 9], including high inter-observer variability of HER2-evaluation [6, 12, 22–24]. Intra-department concordance rate is fairly good within the experienced HER2-testing centers, but it might be reduced in a less-experienced laboratory. Thus HER2 assessment is suggested to be performed in centers with experienced staff [25, 10, 24]. Tumor heterogeneity may also cause insufficient anti-HER2 therapy, especially with the most problematic equivocal (IHC 2+) cases [12, 26, 27, 22].

Our routine immunostaining based assessment validated the automated *MembraneQuant software* application, as a routine semi-quantitative method in a larger set of breast cancers. According to our data, Cohen's kappa revealed an almost perfect agreement (κ=0.872, 95%CI=0.805–0.939) between *Consensus Score* and *MembraneQuant*. Spearman rank-correlation also provided a highly significant correlation (0.928, df=99, $p<0.0001$, 95 % CI for rho 0.904–0.946), thus, we consider *MembraneQuant* application suitable for everyday practice of an appropriately trained pathologist. Furthermore, quadratic weighted kappa values (testing the clinical relevance of results: HER2+ve versus HER2 -ve) showed almost perfect correlation (κ=0.967, 95%CI=0.947–0.986), which validates *MembraneQuant* as a robust platform for application in the clinical decision making.

Table 3 Agreement of FISH and IHC cases regarding Consensus and MembraneQuant Scores

HER2 protein expression by IHC	HER2 testing by FISH n (%)	
	Amplified	Non-amplified
Consensus score Equivocal (2+), *n*=12	6 (50)	6 (50)
Consensus score Positive (3+), *n*=23	23 (100)	0 (0)
MembraneQuant Equivocal (2+), *n*=12	6 (50)	6 (50)
MembraneQuant Positive (3+), *n*=23	23 (100)	0 (0)

Half of the IHC 2+ cases showed amplification of *HER2-gene*, while all IHC 3+ cases had amplification of *HER2-gene*

Our results are in accordance with more non-interventional surveys performed in order to compare the traditional visual-microscopic assessment with semi-automated software-evaluations [28–31]. These results encourage pathologists for using automated HER2 scoring, especially for the challenging equivocal HER2 IHC 2+ cases, where misinterpretation might be partly due to our exhaustion, declining attention. Automated methods offer consistency of evaluation by ensuring the reproducible performance on digital slides with a standard scoring scheme saved as a microscopic image segmentation file (*MembraneQuant; misp*). This can be later uniformly used for all samples for avoiding scoring discrepancies, which potentially result in inappropriate patient selection.

MembraneQuant generates a field score by summarizing scores calculated on individual tumor cells, which can be converted to proportional and intensity scores according to any alternative HER2-scoring scheme. For example, comparing the various direct and derived datasets and H-Scores of our 2+ cases we found significant differences between FISH+ve and FISH-ve cases [32]. Currently, we are running reliability calculations on digital immunostaining signal assessment for predicting the response rate to anti-HER2 therapy.

In conclusion, our study of more than 200 breast cancer samples successfully validated *MembraneQuant* automated and standardized immunostaining evaluation software as a robust and clinically relevant tool producing high correlation values with empirical HER2 evaluation. Improved consistency and accuracy may allow better selection of FISH positive cases from the intermediate category based on immunohistochemistry, which improves patient selection for anti-HER2 therapy. Computer aided marker evaluation can reduce our workload and error-rate and allows standardization and archiving of evaluation results for later reassessment, which also supports quality assurance initiatives in diagnostic pathology.

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Conflict of Interest Tamás Micsik and Tibor Krenács are external advisors of 3DHISTECH Ltd. Gábor Kiszler, Csaba Hegedüs, Dániel Szabó are working at 3DHISTECH Ltd. László Krecsak was employed at 3DHistech in 2008-2010. Béla Molnár is the CEO of 3DHistech.

Contribution Tamás Micsik has planned the study, performed manual and digital scoring and wrote manuscript. Tibor Krenács performed manual scoring and wrote manuscript. Gábor Kiszler, Csaba Hegedüs, Dániel Szabó developed and described MembraneQuant software. László Krecsak performed statistical analysis and wrote manuscript. Béla Molnár designed study and edited manuscript.

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