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ORIGINAL ARTICLE

Free digital image analysis software helps to resolve equivocal scores in HER2 immunohistochemistry

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Abstract Evaluation of human epidermal growth factor receptor 2 (HER2) immunohistochemistry (IHC) is subject to inter-observer variation and lack of reproducibility. Digital image analysis (DIA) has been shown to improve the consistency and accuracy of the evaluation and its use is encouraged in current testing guidelines. We studied whether digital image analysis using a free software application (ImmunoMembrane) can assist in interpreting HER2 IHC in equivocal 2+ cases. We also compared digital photomicrographs with whole-slide images (WSI) as material for ImmunoMembrane DIA. We stained 750 surgical resection specimens of invasive breast cancers immunohistochemically for HER2 and analysed staining with ImmunoMembrane. The ImmunoMembrane DIA scores were compared with the originally responsible pathologists' visual scores, a researcher's visual scores and in situ hybridisation (ISH) results. The originally responsible pathologists reported 9.1 % positive 3+ IHC scores, for the researcher this was 8.4 % and for ImmunoMembrane 9.5 %. Equivocal 2+ scores were 34 % for the pathologists, 43.7 % for the researcher and 10.1 % for ImmunoMembrane. Negative 0/1+ scores were 57.6 % for the pathologists, 46.8 % for the researcher and 80.8 % for ImmunoMembrane. There were six false positive cases, which were classified as 3+ by ImmunoMembrane and negative by ISH. Six cases were false negative defined as 0/1+ by IHC and positive by ISH. ImmunoMembrane DIA using digital photomicrographs and WSI showed almost perfect agreement. In conclusion, digital image analysis by ImmunoMembrane can help to resolve a majority of equivocal 2+ cases in HER2 IHC, which reduces the need for ISH testing.

Keywords Computer-assisted image analysis · Digital pathology · Whole-slide imaging · HER2 · Immunohistochemistry · Breast cancer

Introduction

The human epidermal growth factor receptor 2 (HER2, ERBB2) oncogene protein is overexpressed in approximately 15 % of primary breast cancers [1–4]. The HER2 status of a tumour provides both prognostic and predictive information and is required for patients to qualify for chemotherapy with anti-HER2 drugs such as trastuzumab, lapatinib and pertuzumab [5, 6]. HER2 testing is standard of care in the histopathological diagnosis of breast and gastric cancers and is done mostly by immunohistochemistry (IHC) and in situ hybridisation (ISH) [7, 8].

The optimal strategy for HER2 testing has been under debate for over a decade and there is no consensus as to which testing algorithm is the "gold standard". ISH (both fluorescent and bright-field, to detect HER2 gene amplification) is generally considered accurate and reliable although high reagent costs and labour-intensiveness limit its use in most laboratories to a secondary test to confirm equivocal immunohistochemistry results [7, 9].

HER2 immunohistochemistry is straightforward and can be performed in all modern diagnostic pathology laboratories. Several reagent kits, such as HercepTest™ (Dako, Denmark), PATHWAY™ (Ventana Medical Systems) and Oracle™ (Leica Biosystems) approved for clinical use by the U.S. Food and Drug Administration (FDA), have been analytically

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validated and can be used in existing automated immunostaining devices. However, evaluation of the HER2-stained slides is subject to substantial interobserver variation and lack of reproducibility [7]. In order to improve the accuracy of HER2 testing, the American Society of Clinical Oncology and the College of American Pathologists published [7] and subsequently updated [10] guideline recommendations for determining HER2 status of breast cancer (ASCO/CAP guidelines). According to current recommendations, equivocal results by IHC (staining of 2+ in the four-tier scale of 0 to 3+) require additional confirmation by a validated assay for HER2 gene amplification [10].

Some studies report false scores in up to 14 % of cases [11, 12]. When viewing hundreds of slides (e.g. for a scientific study), most pathologists find it relatively easy to maintain a reproducible visual scale for their 0/1+, 2+ and 3+ scores, whereas in a diagnostic pathology setting, many general pathologists review only a small number of breast cancer cases per week. A pathologist might not see a true 2+ case in weeks to months, making it difficult to score borderline cases reproducibly. While an incorrect HER2 score must be avoided, most pathologists stay on the safe side, give a 2+ score and submit the case for ISH testing. This reduces the cost- and time-effectiveness of the two-step testing algorithm and some authors argue for using FISH rather than IHC as the primary assay for HER2 testing [13].

Digital image analysis (DIA) has been shown to improve the consistency and accuracy of HER2 evaluation by IHC [14, 15] and its use is encouraged in the ASCO/CAP guidelines for cases with 1–2+ IHC staining [10]. We have previously introduced the ImmunoMembrane software for digital image analysis of HER2 IHC using photomicrographs [16]. ImmunoMembrane (http://jvsmicroscope.uta.fi/software) is both a free web application and an open-source plug-in for the public domain image analysis tool ImageJ [17]. Furthermore, ImmunoMembrane has been introduced recently in a web-based whole-slide image viewing system (JVSwebserver, Jilab Inc., Tampere, Finland) which allows its use directly on whole-slide images (WSI). ImmunoMembrane analyses the completeness and intensity of the cell membrane staining reaction, based on the IHC interpretation criteria of the ASCO/CAP guidelines [7, 10]. This approach of analysing the anatomical localisation of the staining reaction rather than just the immunoreaction intensity has been shown to correlate well with gene amplification in HER2 diagnostics [18]. Similar image analysis applications of HER2 have been described by both academic research groups [15, 19–22] and commercial vendors (reviewed elsewhere [23, 24]).

We conducted the current study to test whether ImmunoMembrane can assist in interpreting the HER2 status of cases visually classified as equivocal (2+) by IHC. We also investigated the agreement between digital photomicrographs and WSI as material for ImmunoMembrane DIA.

Materials and methods

A database search was conducted at the Department of Pathology, Helsinki University Central Hospital, Finland, in order to identify invasive breast cancer cases tested for HER2 from the period of 1 January 2010 to 1 July 2011, resulting in 1249 cases. The slides for the corresponding surgical resection specimens were retrieved from the archives, and starting from the earliest case, 750 consecutive cases (one slide per case) were included in the study. The 750 cases represented the period of 1 January 2010 through 23 May 2011, in which period 1186 breast cancer cases were histologically diagnosed. The missing 436 cases were not found in the archives at the time of retrieval and were distributed along the whole period of time taken for the study. The largest number of consecutive cases missing was 28, representing a period of 14 days. The specimens had been routinely fixed for a period of 24–48 h in neutral formalin and embedded in paraffin.

Immunohistochemistry had been performed according to the manufacturer's instructions using the BenchMark XT automated staining system (Ventana Medical Systems, Tucson, AZ) with PATHWAY anti-HER2/neu (4B5) rabbit monoclonal antibody at a dilution of 6 μg/ml and Ventana ultraView Universal DAB Detection Kit (both Ventana Medical Systems). The slides were counterstained using Ventana Hematoxylin II (Ventana Medical Systems) as part of the automated staining procedure.

The HER2 status of the specimens was established by immunohistochemical staining, and positive (3+) and equivocal (2+) cases were further subjected to in situ hybridization to classify them into positive and negative with regard to HER2 gene amplification. A total of 30 pathologists including residents and specialists scored the cases. Six of the specialists were experienced breast pathologists and the residents routinely consulted senior pathologists before giving their scores.

In situ hybridization had been carried out in the BenchMark XT automated staining system using the INFORM HER2 DNA and the INFORM Chromosome 17 probes and the ultraVIEW SISH Detection Kit (all Ventana Medical Systems) according to the manufacturer's instructions. Consecutive sections of the specimens were hybridised with the probes for HER2 and chromosome 17, respectively, and the specimens' HER2 gene status was classified as amplified if the ratio of HER2 to chromosome 17 was over 2.2. Both the ISH and IHC assays have been subjected to external quality assessment by the Nordic Immunohistochemical Quality Control [25].

For this study, the immunohistochemically stained slides were digitally photomicrographed for image analysis and visually scored as positive (3+), equivocal (2+) or negative (0/1+) by the researcher (HOH) without knowledge of the original pathologist's score. Before the photomicrography and visual scoring, the researcher received training in HER2

IHC evaluation from an experienced breast pathologist with whom he also underwent interobserver testing on 35 consecutive cases from the study material. The cases were scored visually as positive (3+), equivocal (2+) or negative (0/1+), without knowledge of the original scores. This yielded a substantial interobserver agreement with a weighted kappa value of 0.71 (95 % confidence interval 0.49–0.94) calculated using linear weighting.

Digital photography was carried out with a standard Olympus microscope equipped with an Mshot MC-30 3.3-megapixel CCD microscope camera (Micro-shot Technology, Guangzhou, China) using a 1X phototube. A minimum of four non-overlapping jpg images with a resolution of 2048 × 1536 pixels was captured per slide using a ×10 objective lens. The photographed areas were chosen to represent the different HER2 staining patterns of the cancer. In cases where the cancer was small and four non-overlapping images could not be captured, the maximum number of non-overlapping images was captured instead. In cases with a large cancer and a wide range of staining patterns, more than four images were captured. A blank field image and an image of the on-slide positive control tissue (3+) were captured for each photography session.

The images were analysed with the ImmunoMembrane software (http://jvsmicroscope.uta.fi/immunomembrane/) using the advanced mode. This permits the user to define custom cutoff values for the IM score (0–20 points: 0–10 points for membrane completeness and 0–10 points for membrane intensity) the software produces and uses to classify staining into 0/1+, 2+ and 3+. We chose cutoff values of 4 and 10 points (i.e. 0–3 points being classified as 0/1+, 4–9 points as 2+ and 10–20 points as 3+) based on our previous experience and empirical testing. The blank field image was used to correct for the microscope illumination and colour balance whereas the positive control image was used for reference contrast and intensity. A stage micrometre was used to calculate the image scale in pixels per micrometre.

The statistical analyses were performed using MedCalc for Windows, version 15.6.1 (MedCalc Software, Ostend, Belgium).

Comparison of photomicrographs and WSI as material for digital image analysis

The records of a HER2 diagnostics reference laboratory (Jilab Inc., Tampere, Finland) were searched retrospectively from May 2015 so as to identify invasive breast cancer cases immunohistochemically stained for HER2. Ten consecutive cases each of IHC staining patterns, 0/1+, 2+ and 3+ according to the original pathologist's report, were identified. The 30 cases, one glass slide per case, were scanned as whole-slide images using the Objective Imaging Surveyor with Turboscan (Objective Imaging Ltd., Cambridge, UK), using a ×20 Plan Apo microscope objective (scanning resolution 0.23 μm per pixel). Whole-slide images were stored as JPEG 2000 images.

The researcher analysed the virtual slides using ImmunoMembrane directly in the whole-slide viewing interface. This was done by defining a polygonal region of interest (ROI) which was analysed by ImmunoMembrane included in the WSI viewer software. A minimum of four areas were analysed per WSI. The ImmunoMembrane DIA results were compared to the original pathologists' results which had been obtained with the help of ImmunoMembrane using digital photomicrographs. In all cases, the HER2 IHC score in the original pathology report matched the ImmunoMembrane score obtained using photomicrographs.

Results

In the original database search result of 1249 cases, 157 cases (12.6 %) were positive by ISH and of the 750 case subset in our current study 74 (9.9 %). In situ hybridisation had been performed in 314 (41.9 %) cases (originally 2+ and 3+ by IHC). The frequencies for a positive (3+) IHC staining for the original pathologist, the researcher and ImmunoMembrane were 8.4 % (63 cases), 9.5 % (71 cases) and 9.1 % (68 cases), respectively. Equivocal (2+) staining was reported by the pathologist, researcher and ImmunoMembrane in 34 % (255 cases), 43.7 % (328 cases) and 10.1 % (76 cases), respectively. Negative staining (0/1+) showed, for the pathologist, researcher and ImmunoMembrane, frequencies of 57.6 % (432 cases), 46.8 % (351 cases) and 80.8 % (606 cases), respectively. These data are summarised in Table 1.

Of the 750 cases, 6 (0.8 %) were false positives in ImmunoMembrane DIA, defined as cases 3+ positive by IHC but negative by ISH. The corresponding number of false positives was for the researcher 8 (1.1 %) and for the pathologist zero. Similarly, there were six (0.8 %) false negative cases by ImmunoMembrane DIA (0/1+ negative by IHC while positive by ISH). The number of false negatives was zero for the researcher whereas it could not be defined for the pathologist because the laboratory performed ISH only on cases originally scored as 2+ or 3+ by IHC. A cross tabulation comparing the IHC scores of the pathologist, researcher and ImmunoMembrane DIA with in situ hybridisation is presented in Table 2. Examples of images from the ImmunoMembrane analyses are shown in Fig. 1.

Analysis of outliers

We categorised the six false positive and six false negative cases (each representing 0.8 % of the total 750 cases) in ImmunoMembrane DIA into four categories: truly discrepant IHC and ISH, borderline IM-score, heterogenous staining, and low-contrast staining. Of the six

Table 1 Frequencies of the HER2 IHC scores in percent, number of cases in parentheses; total number of cases 750

	Pathologist visual score	Researcher visual score	ImmunoMembrane DIA score
Negative (0/1+)	57.6 (432)	46.8 (351)	80.8 (606)
Equivocal (2+)	34 (255)	43.7 (328)	10.1 (76)
Positive (3+)	8.4 (63)	9.5 (71)	9.1 (68)
Total	100 (750)	100 (750)	100 (750)

DIA digital image analysis

false positive cases, only two exhibited true discrepancy between IHC and ISH after review by an experienced breast pathologist, who scored the cases as 3+ positive in line with ImmunoMembrane while ISH was negative. In both cases, the original pathologist had scored the cases 2+ and the researcher 3+.

Two false positive cases showed a borderline IM score of 10 (in the scale of 0–20) which according to our cutoff value qualifies them as positive, although they were negative by ISH. In both cases, both the original pathologist and the researcher had scored the cases 2+.

Two false positive cases were found to exhibit heterogenous staining when reviewed by an experienced breast pathologist. In these cases, some of the photomicrographs were classified by ImmunoMembrane in agreement with ISH whereas a greater number of images showed a negative score, which rendered the overall score in disagreement with ISH. One of the cases was scored 2+ by the original pathologist and 3+ by the researcher whereas the other case was scored 2+ by both.

Of the six false negative cases, one was found to be discrepant between IHC and ISH after review by an experienced breast pathologist (0/1+ IHC-negative in both the pathologist and ImmunoMembrane DIA but positive by ISH). The case was scored 2+ by both the original pathologist and the researcher. Heterogenous staining was observed in three false negative cases. All three cases were scored 2+ by both the original pathologist and the researcher. Two false negative cases exhibited low-contrast staining when reviewed by an experienced breast pathologist. Both cases were scored 2+ by the original pathologist and the researcher.

ImmunoMembrane analysis using digital photomicrographs and whole-slide images

Table 3 presents a cross tabulation of HER2 IHC scores rendered by a pathologist with the help of ImmunoMembrane using digital photomicrographs and by the researcher using ImmunoMembrane directly on scanned whole-slide images. Of the total 30 cases, two were discordant, the first having been scored 2+ using ImmunoMembrane with photomicrographs and 3+ using WSI and the second vice versa (3+ using WSI and 2+ using photomicrographs). Interobserver variability, calculated using linearly weighted kappa statistic, was almost perfect at 0.92 (95 % confidence interval 0.82–1.0). Figure 2 shows ImmunoMembrane being used directly in a WSI viewer.

Discussion

In this study we, show that ImmunoMembrane [16], a free and publicly available cross-platform compatible ImageJ plug-in and web application for digital image analysis of HER2 IHC (http://jvsmicroscope.uta.fi/immunomembrane/), can assist in interpreting the status of cases visually classified as equivocal (2+). In our material of 750 surgical resection specimens, the proportion of immunohistochemical cases classified as equivocal by the original pathologist was 34 % (255 cases), which is on the upper end of the range reported in the literature. In a systematic review from 2007, the mean frequency of an equivocal IHC score in 17 studies was 23.3 % (with a range of 2 to 87.5 %) [26]. In another systematic review from 2009, the mean frequency of an equivocal IHC score in 10 studies was 18.9 % with a range of 7.9 to 53.2 % [27].

Table 2 Cross tabulation comparing HER2 ISH (presence/absence of amplification) with the IHC score given by the pathologist, researcher and ImmunoMembrane digital image analysis (DIA)

	Pathologist visual IHC			Researcher visual IHC			ImmunoMembrane DIA IHC			Total
	0/1+	2+	3+	0/1+	2+	3+	0/1+	2+	3+	
No ISH	427	5	4	322	110	4	424	7	5	436
ISH−	5	235	0	29	203	8	176	58	6	240
ISH+	0	15	59	0	15	59	6	11	57	74
Total	432	255	63	351	328	71	606	76	68	750

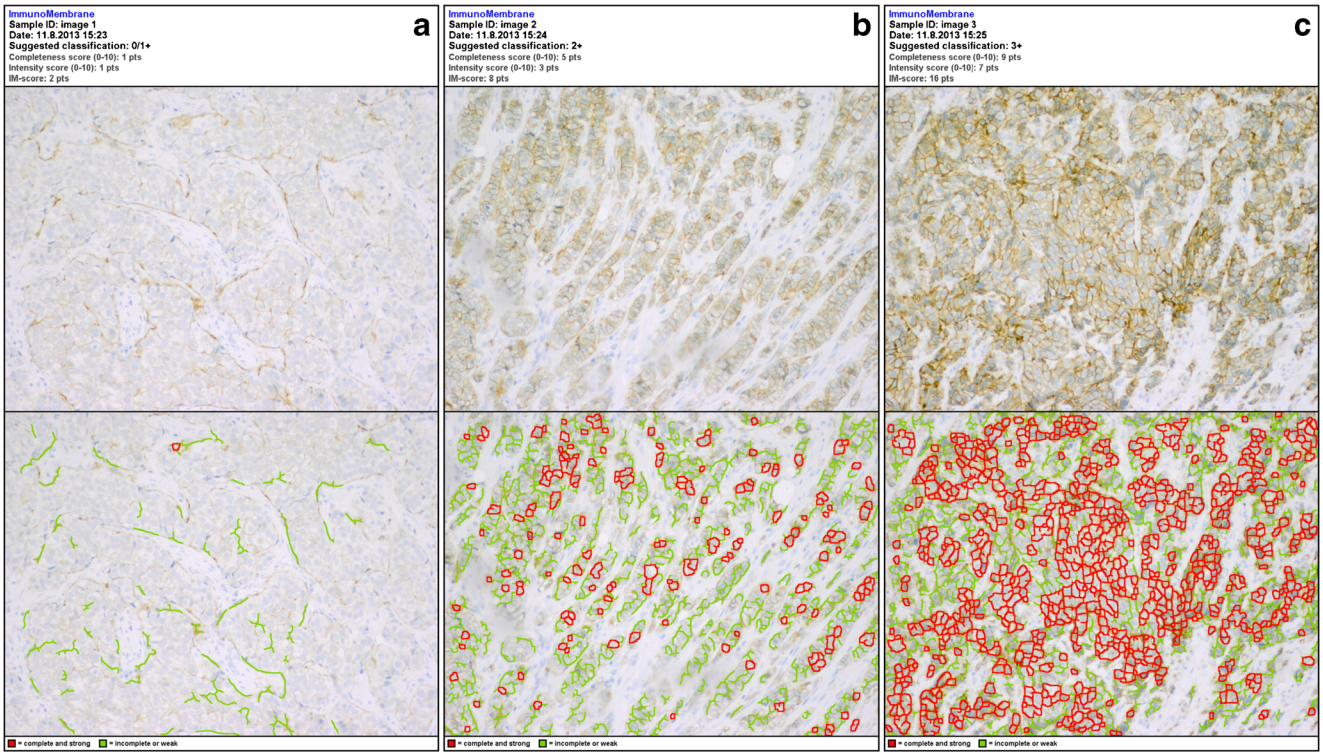

Fig. 1 **a**–**c** Three result images from ImmunoMembrane digital image analysis using photomicrographs. All three cases were originally scored as equivocal (2+) by a pathologist. The first image (**a**) receives an IM score of 2 points and is thus classified as negative (0/1+), the second image (**b**) 8 points (classified as equivocal, 2+) and the third image (**c**) 16 points (classified as positive, 3+). The first two cases were classified visually as 2+ by the researcher and the last one 3+. The first two cases are negative by ISH and the last one positive

The proportion of equivocal results of 34 % (255) by the original pathologist was reduced to 10.1 % (76 cases) by ImmunoMembrane DIA. Of the 255 cases originally classified as equivocal, ImmunoMembrane was able to resolve 190 (74.5 %), 10 of which emerged as positive (3+) and 180 as negative (0/1+). In the ImmunoMembrane analysis, 65 cases remained equivocal, while ImmunoMembrane scored five and six cases as equivocal, originally scored as 3+ and 0/1+, respectively. These data are in line with previous findings of image analysis reducing the proportion of equivocal scores in HER2 immunohistochemistry [15, 20, 28, 29] although there have been opposite findings also, with image analysis increasing the proportion of equivocal results [30, 31]. Images of the result of ImmunoMembrane DIA, demonstrating its discriminative power in cases originally classified as equivocal, are presented in Fig. 1.

Table 3 Cross tabulation comparing ImmunoMembrane digital image analysis (DIA) used with photomicrographs and with whole-slide images

ImmunoMembrane DIA with photomicrographs	ImmunoMembrane DIA with whole-slide images			
	0/1+	2+	3+	Total
0/1+	10	0	0	10
2+	0	9	1	10
3+	0	1	9	10
Total	10	10	10	30

In the two-step testing algorithm proposed in the ASCO/CAP guidelines, in which an equivocal result by IHC requires additional confirmation by a validated assay for HER2 gene amplification, a reduction of the proportion of equivocal cases both increases the information value of IHC and improves the cost- and time-effectiveness of the testing.

Because of the substantial clinical, economic and safety implications of anti-HER2 therapy, assessment of the HER2 status of a tumour must be accurate and reproducible [6, 7]. In our study, we used ISH as the reference method and defined six false positive (3+ IHC-positive and ISH-negative) and six false negative cases (0/1+ IHC-negative and ISH-positive) in the ImmunoMembrane analysis. ISH data was available for 41.9 % of the cases (314/750) because of the laboratory practice of subjecting only immunohistochemically equivocal (2+) and positive (3+) cases for ISH. The frequency of both false positive and false negative cases was thus 1.9 % (6/314) of the cases with ISH data.

After carrying out the present study the laboratory reflex tested for a period of time (8 months, 1 October 2014 through 31 May 2015) all IHC cases (including IHC 0/1+) by ISH (P.

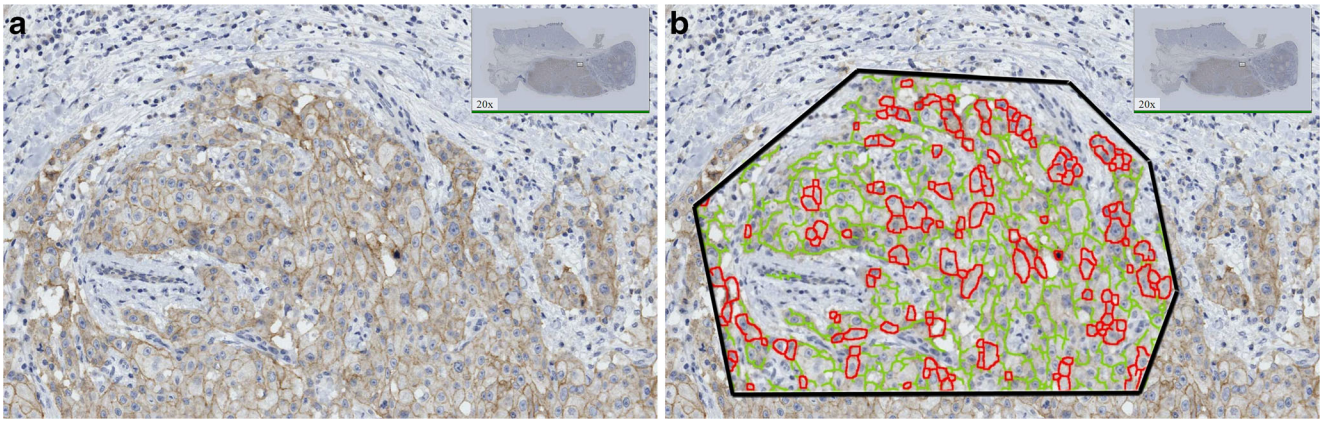

Fig. 2 **a**–**b** Two screenshots demonstrating the process of using ImmunoMembrane digital image analysis directly while viewing a whole-slide image (**a**). The region of interest is demarcated with a *polygon drawing tool*. After the analysis is complete, a pseudo-coloured result image is shown on top of the whole-slide image indicating complete and strong membrane staining in *red* and incomplete or weak staining in *green* (**b**). The depicted tissue exhibits positive 3+ staining according to ImmunoMembrane image analysis

Heikkilä, personal communication, 11 September 2015). In this set of 750 cases, 400 (53.3 %) were scored IHC 0/1+. Only two cases were found positive by ISH. Such a low false negative rate suggests that the proportion of false negatives in our study would not have been substantially affected if the ISH testing had included also the IHC-negative 0/1+ cases. We analysed one of the two false negative cases by ImmunoMembrane DIA and the result was 0/1+, in concordance with the original pathologist score.

The false positive cases by ImmunoMembrane DIA would not have affected anti-HER2 treatment eligibility of the patients because all IHC 2–3+ cases were subjected for ISH. The false negative cases would have made six patients ineligible for anti-HER2 treatment. The false negative rate of 1.9 % is nevertheless lower than the corresponding rate of 6 % reported for an FDA-approved system [29]. It is also lower than the pooled false negative rate of 11 % recently reported for visual scoring, using approved and validated in vitro diagnostic tests in a Nordic IHC quality control programme [32]. However, given the far-reaching clinical and economic consequences of inaccurate HER2 testing [32], even low false negative rates should not be neglected. In the current study, ImmunoMembrane was operated by a researcher; however, as stated in the ASCO/CAP guidelines, in clinical practice, a pathologist must confirm the image analysis result [10].

Discrepancy between IHC analysed by ImmunoMembrane and ISH can be due to several factors. In addition to inaccuracy in the ImmunoMembrane analysis, the analysed images might not be representative of the whole tumour, ISH might not be 100 % accurate either, and regarding false negative cases, some immunohistochemically negative tumours might exhibit HER2 amplification, as is well documented [26, 27, 33]. A borderline score obtained with ImmunoMembrane DIA should be interpreted with care by a pathologist. Heterogenous staining has recently been shown to play a role in discordances between pathologists in reading of HER2 IHC and can also affect the result of ImmunoMembrane image analysis [34]. We propose to analyse a large enough number of image fields to be sure to representatively cover all staining patterns of the specimen in cases with heterogenous staining. This approach is in line with what is recommended in the literature for ISH [35]. Low-contrast staining which can be due to the tissue section properties can influence ImmunoMembrane DIA even with on-slide control tissues.

Our material consisted of a sample of surgical resection specimens from a large university hospital pathology department, reflecting the daily work of diagnostic pathologists. We analysed whole sections instead of tissue microarrays or core needle biopsies in order to mimic, as closely as possible, the daily diagnostic setting. The rate of ISH-positive cases was somewhat low in our material, which can be explained by sampling error due to cases missing from the archives at the time of retrieval. The rate of ISH-positive cases in the original database search is in line with recent findings in the literature [1–4].

As described earlier [16], ImmunoMembrane is a software application for semi-quantitative classification of HER2 IHC, designed as a diagnostic aid for the trained pathologist. ImmunoMembrane does not offer true quantitation of the IHC stain (or ultimately of the amount of HER2 protein), which, in the case of diaminobenzidine (DAB)-based detection systems widely used in HER2 IHC assays, may prove difficult due to the physical properties of the chromogen [36, 37]. ImmunoMembrane performs object-based image analysis [38] by separating the objects of interest (segmenting DAB-stained cell membranes) from the background and analysing them with regard to completeness and intensity of the staining reaction. ImmunoMembrane thus mimics the visual interpretation of HER2 IHC as defined in clinical guidelines and was initially pre-calibrated to match the visual scoring of an expert pathologist. While the image analysis algorithm itself is automated, the selection of regions of interest (ROI) has to be done visually, preferably by a pathologist.

In conclusion, the accuracy of HER2 IHC can be improved in equivocal cases by the use of digital image analysis carried out by the ImmunoMembrane software. In our material, almost three out of four cases originally classified as equivocal by a pathologist were further classified into either negative or positive without loss of accuracy. ImmunoMembrane is easy to apply in clinical practice by virtue of its usability with various combinations of imaging equipment (microscope and camera) and the possibility to use it over the internet (without software download or installation). The fact that the source code of the software is open adds to its utility in the research setting [39, 40].

A new way to integrate ImmunoMembrane into routine diagnostics comes with the increasing use of whole-slide scanners. When HER2 IHC slides are scanned as whole-slide images, pathologists need not spend time acquiring photomicrographs from the microscope. Defining analysable regions of interest in the WSI viewer is fast and yields almost perfect agreement compared with photomicrographs. This is in line with the general finding of good to superior agreement between glass slide and digital slide diagnoses [41] as well as the specific finding of equivalent results in the interpretation of HER2 IHC when using glass slides and whole-slide images [42]. In summary, digital image analysis such as that of HER2 offers a functionality that conventional work with glass slides does not have. The routine use of digital image analysis on whole-slide images offers the pathologist an enhanced diagnostic tool.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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