Digital validation of prognostic and predictive biomarkers in breast cancer

PhD thesis

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I. Introduction

As a result of the development of tumor biology research, prognostic and predictive biomarkers have become part of routine pathological diagnostics. Prognostic markers refer to the prognosis of the particular disease and therefore affect the activity of the disease treatment. Predictive markers indicate responsiveness to a given therapy. A biomarker often has both predictive and prognostic information as well.

In routine pathology four immunohistochemical stainings are performed with breast cancers and the determination of HER2, estrogen and progesterone receptors, just like Ki-67-based proliferation index is crucial for determining further therapy of the breast cancer patient. HER2 is a membrane protein that gives tumor cells more aggressive behavior while we have effective targeted therapy to prevent rapid progression. Nearly two-thirds of breast cancers are hormone receptor positive and the spread of tumors can be controlled by inhibiting the hormone effect. Although tumors with high proliferation rate grow faster, but also respond better to chemotherapy, while low proliferative tumors grow slowly, but are also less capable to be influenced by chemotherapy.

We decide the therapeutic algorithm for each breast cancer patient based on the above-mentioned four routine imunstainings on multidisciplinary oncology board meetings. Thus, biomarker information of routine signout is essential for the patient's course. We have guidelines for evaluating biomarkers, but the evaluating pathologist has the responsibility for the marker positivity determined for that particular case. Since evaluation

remains fundamentally subjective, larger interobserver discrepancies may occur, which may adversely affect the patient's prognosis or therapy.
We have a number of digital pathology platforms that have been validated for routine pathology. With the development of digital technology, we got several digital image analysis methods to eliminate the influence of the subjective human scoring from these biomarker definition and to standardize, to archive the evaluation process, which can potentially improve personalization of therapy.

II. Aims and objectives

Comparison of scoring results on glass and digital slides

To compare the evaluation results of routine immunostained slides of HER2, Estrogen and Progesteron hormone receptors, performed on traditional microscopic and on digitalized slides.

Validation of DIA-evaluation of immunostained slides

HER2: membranous immunoreactions

To compare the results of the traditional, empirical scoring of slides with the result given by an automated DIA-method. Checking whether further information can be taken from the digital way of analysis of normal immunoslides, especially in association with FISH-positivity.

Validation of a robust nuclear-detection algorithm

Predictive and prognostic biomarkers are usually scored on immunohistochemical- and FISH-slides. Dealing with tumor heterogeneity can be facilitated by whole slide image analysis, which can

be effectively assisted by the design and validation of a robust, reliable and quick-enough nucleus detection algorithm. On the other hand, this DIA-method should rely on realistic computing capacity and capable of a sufficiently fast operation to be able to be used on-line, in the routine praxis.

Examination of hormone receptors

To validate the detection and evaluation of the nuclear signals of estrogen and progesteron hormone immunoreactions by comparing the traditional, empirical Allred scoring with a DIA-method.

Ki-67 Proliferation index

To test and validate the digital way of determining tumors' Ki-67 proliferation index in comparison with traditional scoring. Furthermore, to test the clinical relevance of digital proliferation index.

III. Methods

Routine HER2, estrogen and progesterone hormone receptor and Ki-67 immunoslides of breast cancer cases were digitized with Pannoramic Scanner and Viewer (3DHistech, Budapest, Hungary) and subsequent sub-studies were performed.

In 186 breast cancer patients, the results of HER2, ER and PR evaluation on traditional microscopic and on digital slides were compared between the pathologists (interobserver variance) and within different methods (intermethod comparison) of the same pathologist.

For HER2, the traditional scoring results of the TMA with 107 breast cancer cases by 2 pathologists were compared to the results of the

semiautomatic image analysis provided by MembraneQuant (3DHistech, Budapest, Hungary). FISH positive and negative cases of HER2 IHC 2+ cases were investigated separately to check whether the FISH-results can be predicted by digital, in-depth and more detailed analysis of routine immunoreactions.

25 immuno and 5 FISH slides were used to develop and validate a reliable, real-time nucleus-detection algorithm (CellQuant - 3DHistech, Budapest, Hungary) capable of working on whole slide images with a relatively normal hardware-capacity.

The nuclear immunoreactions of hormone receptors were investigated in 16 invasive breast cancer cases using NuclearQuant (3DHistech, Budapest, Hungary) program and given results were compared to the results of microscopic evaluation.

The empirical Ki-67 proliferation Index of 177 cases of estrogen-positive breast cancer patients were compared to the semi-automatic proliferation index (NuclearQuant determination of Ki-67-positive cells in manual annotations of invasive cancers) and to the fully-automated derived proliferation index (PatternQuant recognition of tumor cells and secondarily Ki-67 determination by NuclearQuant) (3DHistech, Budapest, Hungary).

Statistical analysis of data was performed by SPSS 17.0 for Windows (SPSS Inc., Chicago, USA) and MedCalc for Windows v. 11.2.1.0 (MedCalc Software, Mariakerke, Belgium) and Statistica 12 (StatSoft, Tulsa, OK).

Correlation of different variables was evaluated by Cohen's kappa (Cohen's κ), quadratic weighted Kappa and Spearman rank correlation, which latter two tested the clinical relevance of the variables in a more reliable way. We used the following internationally accepted matching groups: 0-0.2: Weak Match; 0.21-0.4: Moderate Match; 0.41-0.6: Medium Match; 0.61-0.8: Appropriate Match; 0.81-1.00: Almost Perfect Match. The clinical relevance of variables was tested on the Kaplan-Meyer survival curve.

IV. Results

Comparison of scoring results on glass and digital slides

When comparing the traditional empirical evaluation of HER2 immunoslides in our 186 cases done by different pathologists, the κ value was between 0.712 and 0.779, while with digital evaluation it was 0.698-0.722, which is basically considered to be an appropriate match. Comparisons between traditional and digital evaluations have broader, but substantially good, κ-values (0.579-0.820). Quadratic weighted κ-value or the Spearman rank correlation – which represent clinical correlation better – values were always above 0.9, coming into the category of almost perfect match.

In Allred-scoring of estrogen hormone receptor, the interobserver κ values of the conventional mode ranged from 0.456 to 0.645, while of the digital platform interobserver κ values were between 0.532 and 0.633, i.e. they were in the medium to appropriate matching classes. When comparing the two methods, the κ values ranged from 0.484 to 0.767, i.e.

they also showed a moderate and appropriate match. The quadratic weighted κ and Spearman rank correlation were always above 0.9, showing the strength of the clinical relevance of either evaluations.

For progesterone hormone receptors, the κ values ranged from 0,496 to 0,642 for the empirical evaluation, between 0,618 and 0,640 for digital evaluation and between 0.545 and 0.781 for the comparison of the two methods. All values were in the medium or appropriate matching classes. The quadratic weighted κ and Spearman rank correlations, which better reflect clinical relevance, with their values above 0,9 have all fallen into the category of almost perfect matches.

Among the three pathologists involved in the study, P1 showed a better match between digital and conventional evaluation for all three biomarkers, which could reflect P1's deeper experience in digital pathology and the impact of the learning curve.

Validation of DIA-evaluation of immunostained slides

HER2: membranous immunreactions

In our study based on the TMA of 107 breast cancer cases, we compared the HER2 scores of two evaluating pathologists (κ = 0.663-0.668), and their consensus HER2-score to the automated HER2 score of MembraneQuant (3DHistech, Budapest, Hungary) and found almost perfect match (κ-value: 0.872). Comparison of quadratic weighted κ values and Spearman rank correlation also showed almost perfect matches (0.842-0.967).

In a small pilot study of 15 HER2 IHC2+ cases we successfully discriminated FISH-positive and FISH-negative cases upon the detailed

digital image analysis of routine HER2 immunoslides, predicting that more information is available for deeper analysis in digitally derived data.

Validation of a robust nuclear-detection algorithm

The nuclear detection algorithm working on whole slide images was tested on 25 immuno- and 5 FISH-slides. By comparing nearly 45,000 manually and digitally annotated nuclei, the sensitivity rate and a positive predictive value was found to be around 90%. Our optimized algorithm built of available algorithms and onto a medium hardware requirements has achieved better and faster results - independently of resolution and quality of images - than other algorithms available on the market.

Examination of hormone receptors

After the calibration phase, the NuclearQuant module (3DHistech, Budapest, Hungary) provided similar result of Allred-scoring on the nuclear estrogen and progesteron hormone immunoslides to the empirical scoring with human eyes. Cohen's κ value was 0.795, quadratic weighted κ was 0.981 and Spearman's rank correlation was 0.975, which means almost perfect match in most cases.

Ki-67 Proliferation Index

The digital Ki-67 proliferation index was determined in the semi-automated and fully automated ways and both resulted in appropriate matching ($r = 0.622$-0.726) with the traditional, empirical Ki-67 proliferation index when tested on 177 estrogen positive breast cancer patients. When we compared the values of the digital image analysis with the mitotic index determined during the grading of breast cancer, we also found a moderate match ($r = 0.508$).

The clinical relevance of the digital proliferation index was tested on Kaplan-Meyer curves using the earlier (14%) and newer (20%) consensus proliferation index defined in St.Gallen. By dichotomization along both values, the survival curves of patients with high and low proliferation index were significantly separated ($p = 0.016$ and $p = 0.001$).

V. Discussion

Comparison of biomarker-scores of glass and digital slides

Based on results of our and other international studies, the traditional microscopic and digital evaluation of HER2, ER and PR immunoslides proved to be equivalent.

Validation of DIA-evaluation of immunostained slides

HER2: membranous immunreactions

Our findings in line with the literature data - altogether based on almost 11000 cases – demonstrates an alternative to the digital evaluation of HER2 scoring. The digital HER2 evaluation not only matches well with the results of conventional evaluation, but also potentially predicts gene amplification and thus might reduce the need of HER2 FISH reactions.

Validation of a robust nuclear-detection algorithm

By carefully combining various DIA algorithms available in the literature, we succeeded in optimizing a medium-hardware based platform that allows high-throughput, whole slide image analysis of FISH- and IHC-slides and to perform real-time nuclear detection. Our algorithm proved to be more accurate, faster, and also more efficient compared to two competing DIA methods.

Investigation of hormone receptors

Based on our and other studies, nearly 26,000 cases demonstrate that digital image analysis can be effectively used to evaluate hormone receptor positivity. The clinical relevance of fully automatic tumor recognition methods and the distinction of subtler positivity classes needs further investigation.

Ki-67 Proliferation Index

Literature data and our research has demonstrated the ability of DIA to classify breast cancer cases into high and low proliferation prognostic groups through nearly 3,000 cases. Furthermore, the more reproducible, standardized, sufficiently quantifiable DIA methods of digitally quantified Ki-67 proliferation index might improve the up-to-now somewhat uncertain role of Ki-67 and successfully introduce it into the row of the predictive and prognostic markers with reliable clinical relevance.

VI. Conclusion

The main findings and new observations of the doctoral dissertation are as follows:

With analyzing significant number of ER, PgR, HER2 and Ki67 immunoslides of breast cancer patients, we are among the first ones who:

1. have confirmed that traditional microscopic and digital way of scoring of breast cancer prognostic and predictive biomarkers are equal.

2. have successfully validated the automated scoring of HER2 membranous immunoreactions to the conventional method, where the results of semi-automated evaluation of the immunoreactions in the critical 2+ positive group supported the pre-separation of FISH positive and negative cases.

3. have developed and validated a robust, well-reproducible, automated nuclear detection algorithm working on medium hardware based computers capable of using on whole slides images of both IHC- and FISH-slides.

4. have validated the digital analysis of nuclear ER, PR and Ki-67 immunoslides to the manual results and to prove the clinical reliability of the yielded automated results.

VII. Publication list

VII. I.a: Publications related to the dissertation

1. **Micsik T**, Kiszler G, Szabó D, Krecsák L, Hegedűs C, Tibor K, Molnár B.: Computer Aided Semi-Automated Evaluation of HER2 Immunodetection-A Robust Solution for Supporting the Accuracy of Anti HER2 Therapy PATHOLOGY AND ONCOLOGY RESEARCH 21:(4) pp. 1005-1011. (2015); doi: 10.1007/s12253-015-9927-6. PMID: 25788005 IF: 1,94
2. **Micsik T**, Elmberger G, Bergquist AM, Fonyad L: Experiences with an International Digital Slide Based Telepathology System for Routine Sign-out between Sweden and Hungary AIMS Medical Science 2:(2) pp. 79-89. (2015) doi: 10.3934/medsci.2015.2.79
3. **Micsik T**, Kiszler G, Szabó D, Krecsák L, Molnár B: Is HER2 amplification predictable by digital immunohistochemistry? DIAGNOSTIC PATHOLOGY 8:(Suppl 1) Paper S 14. (2013) IF:1,85
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5. Krecsák L, **Micsik T**, Kiszler G, Krenács T, Szabó D, Jónás V, Császár G, Czuni L, Gurzó P, Ficsor L, Molnár B: Technical note on the validation of a semi-automated image analysis software application for estrogen and progesterone receptor detection in

breast cancer DIAGNOSTIC PATHOLOGY 6: Paper 6. 6 p. (2011); doi: 10.1186/1746-1596-6-6. PMID: 21244664 IF: 1,638

6. Krenacs T, Zsakovics I, **Micsik T**, Fonyad L, Varga VS, Ficsor L, Kiszler G, Molnár B: Digital microscopy - the upcoming revolution in histopathology teaching, diagnostics, reserach and quality assurance In: Méndez-Vilas A, Diaz J (szerk.) Microscopy: Science, Technology, Applications and Educations. Vol.2. Badajoz: Formatex Research Center, 2010. pp. 965-977. (ISBN:978-84-614-6190-5)

VII. I.b: Oral presentations related to the dissertation

7. **Micsik T**, Kiszler G, Szabó D, Krecsák L, Krenács T, Levente F, Molnár B: Automated image analysis for diagnostic and predictive histopathology; Invited speaker and poster at 23th European Congress of Pathology, Helsinki, Finland 27. August to 1 September 2011.
8. **Micsik T**: Digital evaluation of immunohistological and FISH slides. Oral Presentation at Technology Transfer in Diagnostic Pathology 7th Central European Regional Meeting, Siófok, Hungary, 2012 május 14-16.
9. **Micsik T**, Krenács T, Sápi Z, Turányi E, Krecsák L, Kiszler G, Molnár B: PatternQuant supported Image Analysis for IHC quantification. Oral Presentation at 12th European Congress on Digital Pathology, Paris, France, 2014.06.18-21.
10. **Micsik T**: The role of digital pathology in the evaluation of immunohistochemical results, Oral presentation on the Technology

Transfer in Diagnostic Pathology, 8th Central European Regional Meeting, June 18-19, 2015, Budapest, Hungary

11. **Micsik T**: Prediktív és prognosztikai markerek digitális értékelése/validálása, Felkért előadás 72. MPT konferencia, 2015. 09.24-26. Hajdúszoboszló, Magyarország

VII. II. Other publications

1. Ács B, Madaras L, Kovács KA, **Micsik T**, Tőkes AM, Győrffy B, Kulka J, Szász AM: Reproducibility and Prognostic Potential of Ki-67 Proliferation Index when Comparing Digital-Image Analysis with Standard Semi-Quantitative Evaluation in Breast Cancer. PATHOLOGY AND ONCOLOGY RESEARCH 24:(1) pp. 115-127. (2018) doi: 10.1007/s12253-017-0220-8. PMID: 28401450 IF: 1,736
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