PhD Thesis

Actualities in breast cancer HER2 diagnostics

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1. INTRODUCTION

The c-erbB-2 proto-oncogene is located on chromosome 17 (17q12-21.32), and encodes a 185-kd transmembrane protein. It is a member of the tyrosine kinase growth factor receptor family, however it does not have its own ligand. It is believed to form heterodimers with other members of the receptor family; thus taking part in the cellular response to extracellular proteins such as epidermal growth factor.

The over-expression of this protein, human epidermal growth factor receptor-2 (HER2, HER2/neu, c-erbB-2) is inversely correlated with time to relapse and overall survival in human malignancies. HER2 is amplified and over-expressed in approximately 15-30% of breast cancers. In approximately 90% of these carcinomas, HER2 protein over-expression is attributable to gene amplification. The over-expression of HER2 as detected by immunohistochemistry (IHC) is associated with poor prognosis, and C-erbB-2 abnormalities were also found to be a resistance factor against hormonal therapy and fluorouracyl based chemotherapy; however, it was trastuzumab (Herceptin), a monoclonal antibody directed against the external domain of the HER2 protein that has put HER2 into the centre of attention and investigation. Selection of patients that could benefit from trastuzumab therapy is important not only because of treatment expenses, but also because of the increased myocardial toxicity observed by patients treated with trastuzumab.

Measuring HER2 protein expression by immunohistochemistry is the most commonly used method in routine practice because of ease of

performance and low costs. IHC is widely used for assessing HER2 protein expression on formalin-fixed, paraffin-embedded samples of breast cancer, and is a preferred method for screening and determining which cases need to be evaluated genetically. According to current concepts strong immunohistochemical membrane staining determines HER2 positivity by itself. On the commercial market approximately 20 different IHC antibodies are available. Among them only a few well characterized, FDA (Food and Drug Administration, USA) approved and rather high priced pharmacodiagnostic kits can be found and a great number of cheaper, but usually less reliable antibodies are also in use - the latter always need to be subjected to validation.

HER2 gene amplification as measured by fluorescence in situ hybridization (FISH) has been shown to be the most reliable predictor of clinical response to treatment with trastuzumab. Immunohistochemistry is inferior concerning reliability and specificity compared to FISH, and in situ hybridization has already been advised for primary assessment of HER2 status in breast cancer patients. However, FISH is more time consuming and expensive than IHC, therefore the number of FISH examinations is usually limited to the cases with HER2 over-expression.

It is important that both FISH and IHC in particular, are to be standardized. Besides keeping to strict protocols and using validated, reliable antibodies and diagnostic kits, the application of tissue microarrays (TMAs) are reported to increase the uniformity of circumstances of both IHC and FISH reactions. When constructing a tissue

microarray multiblock, representative tissue cylinders - ranging from 0.6-3mm in maximum diameter - sampled from different donor blocks, are placed into a pre-holed recipient block according to predetermined co-ordinates. Sections of these multiblocks contain cross-sections of all tissue cylinders, available for immunohistochemistry or in situ hybridization. Therefore, TMAs allow the analysis of hundreds of tumour samples simultaneously on a single microscope slide, while antigen retrieval, reagent concentrations, incubation times, wash conditions and temperatures are identical for each core, resulting in high level of standardization. In addition, as the same quantities of reagents are required to perform the examinations on a TMA slide as on regular large sections, TMAs are considered to be efficient and highly cost-effective.

After Battifora's 'sausage block' and 'checkerboard' methods, tissue microarrays were first reported by Kononen et al., in 1998. Since that time it has been reported by many authors that the use of TMAs has significant advantages over traditional techniques. However, due to the heterogeneity of tumour tissues, the sampled cylinders may not always represent the whole of the tumour. It has been proved that TMAs can reliably be used in histopathological and molecular pathological research, when dealing with statistical proportions, but in routine diagnostics at the patient level, even small inaccuracy is intolerable. Some authors have reported about TMAs as an inter- or intralaboratory validation tool, but to our best knowledge, so far only Sapino et al have published relevant information about application of tissue microarrays in the clinical diagnostic field.

2. AIMS

Our aim was first to investigate whether our TMAs are able to help reliably clinical diagnostic work in breast cancer HER2 diagnostics. Then, with the help of tissue microarrays we examined six different immunohistochemical antibodies (four of which were directed against the internal domain /NCL-CB11, Pathway CB11, HercepTest, Pathway RM-4B5/, and the other two /NCL-CBE356, NCL-CBE1/ were directed against the external domain of the HER2 protein), aimed to determine the current value of immunohistochemical testing. We wanted to identify an IHC method that is suitable for high throughput HER2 screening and is also able to predict gene amplification observed with FISH. Finally, from the collected data a reliable and cost-effective HER2 diagnostic algorithm was to be designed.

3. MATERIALS AND METHODS

3.a Selection of patients

For the validation of our microarrays one hundred and seventy-four consecutive cases of invasive breast cancer diagnosed from January 2005 were selected from the routine files of the Department Of Pathology, Medical and Health Science Center, University Of Debrecen, Hungary.

For the immunohistochemical comparison by adding new cases to our selection, altogether one hundred and ninety-nine randomly selected cases of invasive breast cancer were collected.

Corresponding formalin-fixed, paraffin wax embedded tissue blocks containing tumour material were retrieved with their respective hematoxylin-eosin sections. Slides were reviewed and re-evaluated: one hundred and seventy-four cases of invasive ductal carcinoma, twenty-four cases of invasive lobular carcinoma and one case of mucinous carcinoma were revealed. The data was summarized in tables and tissue microarray blocks were built from all cases.

3.b Tissue microarrays

Tissue microarrays were built as described previously by Kononen et al.. Briefly, representative areas from donor blocks were selected under a light microscope. From the designated zones 3mm thick tissue cylinders were punched with a manual tissue puncher (Histopathology Ltd., Pécs, Hungary) and were then introduced into a premade, 24-hole recipient paraffin block. For the valdiation of our TMAs originally only one, however later two separate cylinders from different regions were taken from every tumour block to decrease the effect of intratumoral heterogeneity, according to Gancberg et al.. Extra cylinders of normal spleen or liver were introduced into each multiblock in order to help future microscopic evaluation.

One of the slides was stained with haematoxylin-eosin in order to assess the appropriateness of the multiblocks. When a core was lost, either new sections were cut or a cylinder of the case was involved again in another multiblock.

3.c Immunohistochemistry

Immunohistochemical examination of the TMA slides were carried out with six different antibodies: clone NCL-CB11, NCL-CBE1, NCL-CBE356 (Novocastra, Newcastle upon Tyne, UK), Pathway CB11, Pathway RM-4B5 (Ventana Medical Systems Inc, Tucson, AZ, USA) and HercepTest (Dako, Glostrup, Denmark), according to each manufacturer's instructions. IHC reactions were automatized using different autostainers. Positive and negative controls were included in all IHC runs. Detailed information about the protocols and antibodies used in our study are shown in table 1.

Table 1. Immunohistochemical antibodies and methods

Antibody (dilutions)	Manufacturer	Epitope retrieval	Chromogene	Automatization
NCL-CB11 (1:40)	Novocastra	pH:6 Citrate Buffer 3' high pressure	DAB	Nexes
Pathway CB11 (RTU)	Ventana	pH:6 Citrate Buffer 3' high pressure	DAB	Nexes
HercepTest (RTU)	DAKO	pH:7.2 Citrate Buffer (0.1 mol/L) 40' 95C waterbath	DAB	DAKO Autostainer
NCL-CBE356 (1:40)	Novocastra	not required	DAB	Nexes
NCL-CBE1 (1:15)	Novocastra	pH:6 Citrate Buffer 3' high pressure	DAB	Nexes
Pathway RM-4B5 (RTU)	Ventana	pH:6 Citrate Buffer 3' high pressure	DAB	Nexes

DAB: 5% 3,3‘-diaminobenzidine tetrahydrochloride chromogen solution
Nexes: Ventana Nexes (Ventana Medical Systems Inc., Tucson, AZ, USA)
DAKO Autostainer (Dako, Glostrup, Denmark)
RTU: ready-to-use

The results were scored according to Sapino et al.. Briefly, the interpretation was performed following substantially the well known FDA approved scoring system designed for HercepTest, based on the membrane staining pattern, intensity and ratio of invasive tumour cells. Immunoreactivity was considered weakly positive (2+) if more than 10%

of the tumour cells showed weak to moderate complete membrane staining, or intensively positive (3+) if more than 10% of the tumour cells showed strong complete membrane staining. When the membrane staining was absent or present in less than 10% of invasive tumour cells (0 or 1+) the IHC reaction was considered negative.

Immunohistochemical reactions with the NCL-CB11 and HercepTest antibodies were repeated also on large sections with the same methods for the validation of tissue microarrays.

2.d Fluorescence in situ hybridization

Four-µm thick TMA sections mounted on sylanized slides were prepared for hybridization using Paraffin Pretreatment Kit II (Vysis, Des Plaines, IL, USA), according to the manufacturer's instructions. After digestion, genomic DNA was codenaturated at 90°C for 10 mins together with spectrum orange labeled HER2 probe and spectrum green labeled chromosome 17 centromeric probe from the Pathvysion HER-2/neu DNA Probe Kit (Vysis). The fluorescent probes were hybridized at 37°C to the genomic DNA overnight. Both codenaturation and hybridization was carried out using the Thermobrite manual FISH platform (Vysis). After nuclear counterstaining with 4',6-diamidine-2'-phenylindol-dihydrochloride (DAPI, Vysis, Des Plaines, IL, USA) slides were examined using a fluorescent microscope (BX51, Olympus, Tokyo, Japan) by first identifying the tissue cores under 10X objective applying the DAPI filter. For studying the nuclei of the tumor cells we applied a 100X objective with immersion

oil, using the DAPI/Orange/Green triple bandpass filter. After summing up the orange and green signals in 40 nuclei of each sample a mean value was taken, and a ratio of the average orange, HER2 signals and the green, chromosome 17 signals was calculated.

A case was considered non-amplified when the HER2/CEP17 ratio was less than 1.8 and amplified when the ratio was more than 2.2. When the calculated ratio was between 1.8-2.2, another 20 nuclei were counted for signals and the ratio was recalculated for the 60 nuclei. When the average number of CEP17 signals per nucleus was between 1.5-2.5 the tumour specimen was considered to be euploid, when it was not more than 1.5 the sample was considered to bear monosomy, and in any other cases the specimen was diagnosed to bear polysomy of the chromosome 17.

3.e Statistics

Comparing the immunohistochemical data obtained from large sections (NCL-CB11 and HercepTest) with the results of the tissue microarray based immunohistochemistry (NCL-CB11 and HercepTest, respectively) we have calculated immunohistochemical concordances, and then confronted IHC data with the results of fluorescence in situ hybridization performed on TMA slides.

Then, using genetic analysis by FISH as the end point, sensitivity, specificity, positive and negative predictive values and overall accuracy were calculated for all six IHC assays. Two different calculations were made: in the first approach dichotomizing IHC results taking both 2+ and

3+ reactions as positive IHC results, while in the second method considering only the 3+ reactions as positives. Immunohistochemically 0 and 1+ cases were marked as IHC negatives in both methods. When dichotomizing the FISH results, cases with monosomy or polysomy of the chromosome 17 were regarded as FISH negative, considering the fact that to present time none of these tumors are expected to respond to trastuzumab therapy equally to those with gene amplification. Cases with HER2 gene amplification were FISH positives.

4. RESULTS

4.a Validation of tissue microarrays

During all staining and washing steps our TMA slides received stress causing 10.2% of the cores to slide off.

With the NCL-CB11 IHC reactions our results were the following. From the 91/174 (52%) routine HER2 negative cases 86 (94.5%) proved to be HER2 negative using tissue microarrays. In 83 cases diploid chromosomal status without amplification, in two cases HER2 gene amplification and in one case aneusomy of the chromosome 17 was revealed by FISH. Among the four TMA HER2 2+ cases three turned out to be diploid, HER2 non-amplified using FISH; in one case aneusomy was revealed by FISH. The case that showed 3+ immunohistochemical reaction on the TMA slide was found to be diploid, non-amplified by FISH.

We found a fairly large number of equivocal (2+) cases of 28% at our routinely processed large sections when IHC was performed with the CB11 clone. From the 49/174 routinely processed HER2 2+ cases 40 (81.7%) proved to be HER2 2+ and two (4%) turned out to be HER2 3+ on tissue microarray slides; the latter cases did not show either gene amplification or aneusomy. The seven (14.3%) non-correlating, TMA HER2 negative cases were all diploid, HER2 non-amplified tumours according to the FISH results. Among the 49 cases there were only eight cases that showed HER2 gene amplification and diploid chromosomal status; they all proved to be HER2 2+ immunohistochemically on the TMA slides.

The remaining 34/174 (20%) routine HER2 3+ cases could also be sorted out into three groups, as above: 29 (85.3%) cases proved to be HER2 3+, two (5.9%) cases HER2 2+ and three (8.8%) cases HER2 negative according to TMA slides. Two from the three non-correlating tumours turned out to be diploid, HER2 non-amplified tumours using FISH, and at one tumour aneuploidy was revealed. Only seven of the 29 tumours, which were both on TMA slides and on routinely processed large sections HER2 3+, were diploid, non-amplified. Twenty-one cases were found to be diploid, HER2 amplified tumours using FISH, and there was one tumour showing aneuploidy without HER2 amplification.

Somewhat different proportions could have been obtained while using HercepTest for immunohistochemical reactions, both at routine and at TMA levels.

One hundread and twenty-three (123/174, 71%) cases showed HER2 negativity on the large sections with HercepTest from which 114 (92.7%) turned out to be HER2 negative also on TMA slides. Among them two cases showed HER2 gene amplification, and two other cases had aneusomy of the chromosome 17. Seven cases (5.7%) including three cases with gene amplification showed 2+ IHC positivity, while two more cases (1.6%) - one of them having HER2 gene amplification - developed 3+ IHC positivity on TMAs.

Only 31/174 cases (18%) exhibited 2+ protein expression on large sections with HercepTest of which 25 cases (85.7%) showed 2+, one case (3.2%) showed 3+ protein expression on TMA slides. Nine of the above 26 concordant cases also showed gene amplification, while all the five (16.1%) discordant cases proved to be diploid, HER2 non-amplified tumours.

Sixteen (16/20, 80%) out of 20 cases with 3+ IHC on large sections was also 3+ immunohistochemically on TMAs, 15 of which also bore HER2 gene amplification, and one showed aneusomy of the chromosome 17. Two cases (10%) exhibited 2+ IHC on TMAs with only one of them being HER2 amplified, while the two remaining cases were HER2 negative with IHC on TMAs; one of them being also negative with FISH, the other one bearing aneusomy of the chromosome 17.

3.b Comparison of anti-HER2 immunohistochemical antibodies

During automated processing of TMA slides we experienced a loss of approximately 8% of the cores.

IHC 3+ protein overexpression was found in 8.14-11.76% of cases, according to which antibody was used. Together with the equivocal (2+) cases the proportion of HER2 overexpressing cases is 11.63-41.18%. The highest rate of 2+ and 3+ cases was found with NCL-CB11, while the lowest was found with RM-4B5. Altogether 25 of the 199 cases showed gene amplification with FISH (12.56%). Only the HercepTest did not show cases without protein expression harbouring gene amplification, and the highest number of such cases (four) were found with CBE1 and Pathway RM-4B5 reactions.

According to the calculations with the first approach, the highest sensitivity and negative predictive value (NPV) were the ones of the HercepTest - both calculated to be 100%, because not having found false negative cases while using HercepTest. The second highest sensitivity was the one of NCL-CB11 (95.65%), while Pathway RM-4B5 turned out to have the smallest sensitivity with 80.00%. The second highest NPV - the one of NCL-CB11 - was less than 1% below the NPV of the HercepTest (99.09%), but even the lowest NPV was just below 97% (96.95% of NCL-CBE1).

Pathway RM-4B5 reached the highest specificity, positive predictive value (PPV) and overall accuracy. Pathway CB11 turned out to be nearly as specific as the HercepTest with a specificity of 89.96%, while NCL-CB11

seems to be the least specific (66.46%) predictor of HER2 gene amplification observed by FISH.

Markedly low PPVs were found at all IHC examinations, except for Pathway RM-4B5 having the highest value of all with 80.00%; nevertheless, the second highest PPV was only slightly higher than the half of the highest value (Pathway CB11– 48.78%), and the lowest PPV was associated with NCL-CB11 (28.57%).

In terms of overall accuracy the most reliable antibody was found to be Pathway RM-4B5 (95.34%), followed by Pathway CB11 with 86.96%. Interestingly, NCL-CB11 - just as with specificity and PPV mentioned above – showed the lowest overall accuracy (70.05%).

When considering 2+ reactions immunohistochemically negative, remarkable changes occured in the ranking of the statistical values of the different IHC antibodies, related to the fact that approximatelly 90% of 2+ cases did not show gene amplification. Changing of the cut-off value, specificities, PPVs and overall accuracies raised convincingly, while sensitivities and NPVs were found to be notably lower.

The two CB11 antibodies showed the highest sensitivity and NPV (NCL-CB11 - 73.91% and 96.36%, respectively), specificity and PPV (Pathway CB11 – 99.38% and 93.75%, respectively). The second highest specificity and PPV were the ones of Pathway RM-4B5 (99.34% and 92.86%, respectively), while the lowest values in these relations also belonged to NCL-CB11 (96.95% and 77.27%, respectively). The HercepTest showed the second highest sensitivity and NPV (70.83% and

96.06%, respectively, instead of 100%); while the lowest sensitivity was the one of NCL-CBE1 (63.63%), and the lowest NPV belonged to the Pathway CB11 (95.24%). The highest overall accuracy was reached by the HercepTest (95.43%), slightly higher than that of Pathway RM-4B5 after statistical calculations with the first approach (see above). When excluding 2+ cases from IHC positives Pathway RM-4B5 still turned out to be the second most reliable antibody, and NCL-CBE356 proved to be the least accurate (93.23%).

5. DISCUSSION

Determination of the HER2 status has become an inevitable step of routine pathological breast cancer diagnostics, not only having prognostic significance, but also being important in therapeutic decision making. Standardization of both immunhistochemistry and the DNA level analysis is crucial in order to gain reliable results. Besides confining ourselves to the application of validated diagnostic kits and strict protocols, the integration of tissue microarrays into the diagnostic algorithm may further improve the reliability of results. Our first aim was to investigate the trustworthiness of our tissue microarrays in routine breast cancer HER2 diagnostics.

Tissue microarrays have been used for a long time in pathological research to improve the performance of IHC and FISH examinations on large investigational populations. Nevertheless, due to the questionable reliability of TMAs, routine diagnostic application of multiblocks has not

been thoroughly examined. Parallel with, but independently from our study group, Sapino and colleagues proceeded an investigation on the routine application of TMAs, however they have completed direct comparison of only 20 cases. In the first part of our study we have examined 174 invasive breast cancer cases parallel on large sections and on TMAs with IHC and FISH. While using both NCL-CB11 and the HercepTest antibodies high concordance was established at HER2 negative (94.5% and 92.7%, respectively), HER2 2+ (81.7% - 2+ and 4% - 3+; 80.7% - 2+ and 3.2% - 3+, respectively) and at HER2 3+ cases (5.9% - 2+ and 85.3% - 3+; 10% - 2+ and 80% - 3+, respectively). According to our results we can claim that our TMAs can be utilized reliably in breast cancer HER2 immunohistochemistry. Having all discordant results between IHC and FISH confirmed by FISH on regular large sections, we may also declare that our TMAs provide reliable FISH results in breast cancer HER2 diagnostics.

We have sampled only one tissue cylinder per tumour, so obviously the reliability of our TMAs can be further improved with sampling more than one cylinder. However, if we accept the results of IHC or FISH examination performed on a breast core biopsy, we may as well simply consider reliable TMA cores that contain an equally small fraction of tumour cells.

A further advantage of TMAs is the restriction of the examiner to small tumour cell rich areas while screening FISH slides, instead of screening the whole large section. A possible disadvantage of TMAs can be

the increased turn-around time for IHC and FISH examinations. Nonetheless, our experience so far has showed that at a certain frequency of breast cancer cases diagnosed, the construction of TMAs did not imply significant extra energy and time that would affect the speed of diagnostics.

When performing IHC with the NCL-CB11, we have observed a relatively high proportion of 2+ cases (28%) that made us repeat the IHC analysis with the FDA approved HercepTest. With this pharmacodiagnostic kit the distribution of IHC negative, IHC 2+ and IHC 3+ cases was closer to the data given in the literature, while the concordances between large sections and TMAs did not differ significantly.

We have found HER2 over-expressing cases without gene amplification and also IHC negative cases with HER2 gene amplification. At these - so called - discordant cases, FISH examination was repeated with the conventional slide-by-slide method, and the original TMA FISH results were confirmed at all cases. The cause of this discordance is possibly some kind of intracellular mechanism leading to impaired or increased internalization of the HER2 protein.

With the NCL-CB11 immunohistochemistry only 62-66% of the IHC 3+ cases were amplified, while with the HercepTest this rate was 80-89%. According to the given data in the literature, the amplification rate of IHC 3+ cases ranges from 55.6% to 100%. This wide variety may call the reliability of immunohistochemistry into question. Proceeding from this

fact, in the second part of our study we have examined six commercially available, anti-HER2 IHC antibodies.

Three FDA approved pharmacodiagnostic kits (HercepTest, Ventana Pathway CB11 and Ventana Pathway RM-4B5), two antibodies driven against the extracellular domain of the HER2 (NCL-CBE1 and NCL-CBE356) and the commonly used antibody specific to the intracellular domain, NCL-CB11 were examined and compared on 199 breast cancer patient. Taking the FISH results as the end point, we have characterized the different IHC methods. Dichotomization of the IHC results was performed twice (IHC negative vs. 2+/3+ and IHC negative/2+ vs. 3+).

However, according to the results presented by Ainsworth et al, we have expected better performance by the extracellular domain specific antibodies (NCL-CBE356, NCL-CBE1). Both the poor quality of membrane staining and the significant background staining have aggravated the interpretation of reactions and neither their specificity, nor their sensitivity was outstanding. Therefore, we recommend a validation process prior to their utilization in routine HER2 IHC.

The most promising antibody was the pharmacodiagnostic Pathway RM-4B5, which was reported to be reliable, specific and sensitive by Powell et al.. We experienced a remarkably high overall accuracy, but also lack of sensitivity associated with the antibody. It should be noted, that aggresive antigene retrieval did not enhance the membrane staining pattern, unlike being subjected to prolonged incubation with the primary antibody. Current automatization, however, does not allow incubation

longer than 32 minutes, thus we proceeded from the original results at evaluation.

If we consider immunohistochemistry as a screening step to select the cases with 2+ and 3+ positivity for further evaluation with FISH, the most important characteristic of IHC is its sensitivity. In this relation, we have found HercepTest and NCL-CB11 prominently suitable. Specificity is also an important characteristic, in particular if IHC 3+ cases do not undergo further genetic analysis. The highest specificity was associated with Pathway RM-4B5 at 2-3+ cases, and Pathway CB11 at 3+ cases. The best membrane staining pattern was provided by HercepTest and Pathway CB11.

The sensitivity of IHC may be further improved if a second immunohistochemical reaction is performed with a different antibody, and gene level examination could be excluded only when both IHC reactions are negative. According to our results we would recommend the HercepTest and one of the CB11 antibodies for the two IHC reactions.

In conclusion, we established a diagnostic algorithm following our results. All breast cancer cases go through a conventional IHC staining with the NCL-CB11 antibody to provide a preliminary result. Then all cases are sampled, and TMAs are constructed. A second immunohistochemical reaction with the HercepTest and fluorescence in situ hybridization are performed on TMA slides. If both IHC reactions correlate with the FISH, we have our final result. If one or both IHC reactions show discordance with the FISH result, a conventional, large slide FISH is performed, and

the result of this reaction is considered the final result. Performing FISH in all cases makes it possible to reduce the loss of not over-expressed, but FISH positive cases. And also, if strongly IHC positive cases do not harbour gene amplification on TMA slides, the result of the genetic analysis is confirmed on conventional large sections.

PUBLICATIONS, AS THE BASIS OF PRESENT WORK

1. **Egervari K**, Szollosi Z, Nemes Z. Tissue microarray technology in breast cancer HER2 diagnostics. Pathol Res Pract 2007;203:169-177. IF: 0.892
2. **Egervari K**, Szollosi Z, Nemes Z. Immunhistochemical antibodies in breast cancer HER2 diagnostics. A comparative immunohistochemical and fluorescence in situ hybridization study. Tumor Biol Accepted for publiation. IF:2.407

OTHER PUBLICATIONS

1. Szollosi Z, Scholtz B, **Egervari K**, Nemes Z. Transformed dermatofibrosarcoma protuberans: real time PCR detection of COLIA1-PDGFB fusion transcripts in sarcomatous areas. J Clin Pathol. 2007;60:190-4. IF: 2.245
2. Szollosi Z, **Egervari K**, Nemes Z, Kaczur V. Re: Lottner et al. simultaneous detection of HER2/neu gene amplification and protein overexpression in paraffin-embedded breast cancer. J Pathol. 2005;207:119-20. IF: 6.213
3. Szollosi Z, Nemeth T, **Egervari K**, Nemes Z. Histiocyte-like cells expressing factor XIIIa do not belong to the neoplastic cell population in malignant fibrous histiocytoma. Pathol Res Pract. 2005;201:369-77. IF: 0.892

4. **Egervari K**, Szollosi Z, Nemes Z, Kaczur V. Comparison of immunohistochemical and fluorescence in situ hybridization assessment of HER-2 status in routine practice. Am J Clin Pathol. 2006;125:155-6. IF: 2.939
5. **Egervari K**, Szollosi Z, Nemes Z. Reply to „IHC for Her2 with CBE356 antibody is a more accurate predictor of Her2 gene amplification by FISH than HercepTest in breast carcinoma". J Clin Pathol. 2006;59:665. IF: 2.245
6. **Egervari K**, Szollosi Z, Nemes Z. Re: A New Rabbit Monoclonal Antibody (4B5) for the Immunohistochemical (IHC) Determination of the HER2 Status in Breast Cancer: Comparison With CB11, Fluorescence In Situ Hybridization (FISH), and Interlaboratory Reproducibility. Appl Immunohist Mol Morphol. Közlésre elfogadva. IF: 1.621

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