

ORIGINAL ARTICLE

HER2/neu Expression and Gene Alterations in Pancreatic Ductal Adenocarcinoma: A Comparative Immunohistochemistry and Chromogenic in Situ Hybridization Study Based on Tissue Microarrays and Computerized Image Analysis

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ABSTRACT

Context HER2/neu overexpression is observed in many cancers including pancreatic ductal adenocarcinoma. Although immunohistochemistry remains the basic method for evaluating HER2/neu protein expression, significant information regarding gene status cannot be assessed.

Design Using tissue microarray technology, fifty histologically confirmed pancreatic ductal adenocarcinomas were cored twice and re-embedded in one paraffin block. Immunohistochemistry (clone TAB 250) and chromogenic (HER2/neu amplification Spot Light kit) in situ hybridization protocols were performed. The immunostained slides were evaluated by conventional eye microscopy and digital image analysis. The chi square test and the kappa statistic were applied by running the SPSS package.

Main outcome measures The levels of staining intensity were estimated by the performance of a semi automated image analysis system.

Results HER2/neu gene amplification was detected in 8/50 cases (16%). Chromosome

17 aneuploidy was detected in 19 cases (38%). Significant improvement in interobserver agreement ($\kappa=0.76$ vs. 0.94) was achieved correlating the immunohistochemical results obtained by conventional eye and digital microscopy, especially in the cases of overexpression (2+, 3+). Finally, 29 (58%), 11 (22%), 6 (12%) and 4 (8%) cases were characterized as 0, 1+, 2+ and 3+, respectively. HER2/neu protein expression was significantly associated with grade ($P=0.019$), but not with stage ($P=0.466$). In addition, chromosome 17 and gene status were not correlated with stage and grade.

Conclusion Our results indicate that a subset of pancreatic ductal adenocarcinomas is characterized by HER2/neu gene amplification. In contrast to breast cancer, protein overexpression does not predict this specific gene deregulation mechanism. This event may reflect the different biological role of the molecule in those two solid tumours, affecting the response to novel targeted agents, such as monoclonal anti-HER2/neu antibodies. Furthermore, evaluation of HER2/neu protein expression based on digital image analysis and not only on conventional eye microscopy improves the accuracy and

reliability of immunohistochemical estimation, although that does not demonstrate clinical significance and prognostic value in pancreatic ductal adenocarcinoma.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive malignancy comprising 80-90% of all neoplasms involving the exocrine pancreas [1]. Extensive cytogenetic analyses have confirmed that pancreatic carcinogenesis is a multistep progressive process involving specific chromosome aberrations and including oncogene and suppressor gene deregulations, such as k-ras or p53 intragenic point mutations, respectively [2, 3]. Similarly, some oncogenes such as c-myc and HER2/neu are activated by amplification [4]. According to many studies, this genetic event is significantly correlated to the aggressiveness of the cancers, such as breast adenocarcinoma (advanced stage, metastasis, poor survival) [5, 6]. HER2/neu protein overexpression is also a crucial event regarding breast cancer because it is strictly associated with gene amplification in the majority of the cases examined and correlated with response to novel targeted therapeutic protocols. [7, 8].

The HER2/neu gene also known as rat neu or c-erb-2, is located on the long arm of chromosome 17 (17q12-21.32) and encodes a 185-kd transmembrane glycoprotein which demonstrates tyrosine kinase activity [9]. This protein belongs to the type I epidermal growth factor receptor (EGFR) family. As in the other members, it is characterized by three distinct structural and functional domains, including an extracellular cysteine-rich section, a transmembrane bridge and an internal cytoplasmic tyrosine-kinase section. Signal transduction to the nucleus is mediated by ligand-binding induced stabilization of receptor dimmers which is followed by receptor autophosphorylation and recruitment of specific SH-2 proteins [10]. Novel targeted therapeutic strategies include chimeric or

recombinant humanized monoclonal antibodies (i.e. trastuzumab) which bind to the extra cellular domain of the receptor preventing excessive signal transduction to the nucleus, resulting from an oncogenic malformation [11, 12].

Although immunohistochemistry (IHC) is a basic procedure for the laboratory assessment of HER2/neu protein expression levels, interpretation of the results is the most critical process. Immunohistochemical analysis is frequently influenced by technical considerations, such as fixation procedures affecting the quality of antigen epitopes in the paraffin-embedded tissues, and methodological differences (IHC protocols) [13]. Selection of specific antibodies and scoring methods also appear to be very important parameters for the accurate evaluation of protein expression. Furthermore, molecular techniques, such as fluorescence in situ hybridization (FISH) and chromogenic in situ hybridization (CISH), have already been developed in order to correlate the protein expression levels with HER2/neu gene status (normal or amplification) [14, 15].

This study was designed to serve two purposes. The first purpose was to correlate IHC and CISH results with biological parameters in PDACs based on tissue microarrays. The second was to compare the results obtained by semi-quantitative (conventional eye-microscopy) and fully quantitative (computerized image analysis) methods evaluating HER2/neu protein expression. To our knowledge, this is the first computerized image analysis study demonstrating a model of evaluation of HER2/neu protein expression in PDAC.

MATERIALS AND METHODS

Study Group

For the purposes of our study, we obtained 50 paraffin embedded tissue samples of histologically confirmed primary PDACs from patients who underwent radical (Whipple) or partial pancreatic resection between 1998 and 2004. Twenty-nine patients

Table 1. Clinicopathological data of 50 patients with pancreatic ductal adenocarcinoma.

| | | |
|----------------------|--------|----------|
| Sex | Male | 29 (58%) |
| | Female | 21 (42%) |
| Tumour origin | Head | 41 (82%) |
| | Body | 5 (10%) |
| | Tail | 4 (8%) |
| Grade | 1 | 10 (20%) |
| | 2 | 20 (40%) |
| | 3 | 20 (40%) |
| Stage | I | 9 (18%) |
| | II | 5 (10%) |
| | III-IV | 36 (72%) |

were male with a median age of 62.5 years (range: 58-67 years) and 21 were female with a median age of 66.8 years (range: 62-71 years). Tissue sections from benign-appearing epithelia, adjacent to malignant tissue were used as a normal control group. The archival samples were fixed in 10% neutral-buffered formalin. Hematoxylin and eosin (H&E)-stained slides of the corresponding samples were reviewed for confirmation of the histopathological diagnosis. All adenocarcinomas were graded and staged according to the "Digestive System Tumours Classification Criteria of the World Health Organization" (WHO 2000). The clinicopathological data are demonstrated in Table 1.

Tissue Microarray (TMA) Construction

The areas of interest were identified on H&E stained slides by a conventional microscope (Olympus BX-50, Melville, NY, USA). The corresponding paraffin blocks were obtained for the construction of one TMA block. Using TMArrayer-100 (Chemicon International, Temecula, CA, USA), all of the source blocks were cored twice in order to increase the reliability of the TMA method and 1 mm diameter tissue cylindrical cores were transferred from each conventional donor block to the recipient block. The final constructed TMA block contained approximately 100 cores (50 pairs) of cylindrical tissue specimens. After 3 mm microtome sectioning and H&E staining, we

microscopically observed that each case was represented by at least one or two tissue spots confirming the adequacy of the corresponding cases (tissue cylinders).

IHC Antibodies and CISH Probes

Ready-to-use HER2/neu monoclonal mouse antibody (clone TAB 250-Zymed/InVitrogen, San Francisco, USA) was selected for the study. This antibody recognizes the extracellular domain of the HER2/neu protein, and does not react with the other erbB receptors. HER2/neu gene status was determined using the ready-to-use Spot Light HER2/neu DNA Probe (Zymed/InVitrogen, San Francisco, USA). This digoxigenin-labeled probe is located on 17q12-21 and covers the entire gene area. Chromosome 17 status was also determined by the ready-to-use biotin-labeled chromosome 17 centromeric probe (Zymed/InVitrogen, San Francisco, USA), recognizing the specific repetitive centromeric DNA sequences known as alpha-satellite DNA.

Immunohistochemistry (IHC) Assay

IHC for HER/neu antigen was carried out on 3 µm paraffin sections of the TMA block described above, using the EnVision protocol (DAKO, Glostrup, Denmark). Those tumor sections were mounted on siliconized glass slides, air-dried and heated at 37°C overnight. The slides were then deparaffinized and rehydrated in graded alcohols. Incubation with an enzyme (ficin) was performed for 10 min. After H₂O₂ inhibition, TAB 250 monoclonal antibody was applied for 40 min at a dilution of 1:70. The slides were incubated with a soluble dextran polymer for 20 min and DAB chromogen was applied for 5 min. Finally, the sections were counterstained using hematoxylin for 1 min, dehydrated in xylene and mounted. The IHC protocol was performed using an automated staining system (I 6000 Biogenex, San Ramon, CA, USA). Membranous staining was considered to be acceptable. Breast cancer tissue sections overexpressing

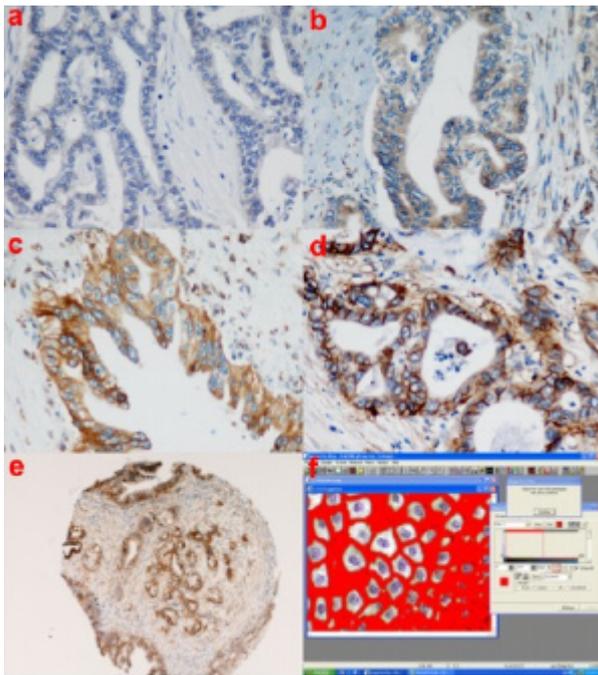


Figure 1. Digital images of different HER2/neu protein expression cases. **a.** score 0; **b.** score 1+; **c.** and **d.** scores 2+ and 3+, according to conventional eye microscopy: image analysis identified borderline, but distinct levels of optical density and, finally, those cases which were characterized as 2+ (**c.**) and 3+ (**d.**), respectively (original magnification: 40x); **e.** a tissue microarray spot (core diameter: 1 mm) stained by HER2/neu monoclonal antibody TAB 250 (original magnification: 2.5x); **f.** image analysis process (red areas represent the values of HER2/neu membranous immunostaining).

HER2/neu (2+, 3+) were used as a positive control for evaluating the stain pattern.

Evaluation of IHC Results

In order to evaluate the IHC results, we performed both conventional eye microscopy and computerized image analysis methods. Levels of HER2/neu protein expression were evaluated semi-quantitatively using Zymed evaluation guidelines, similar to DAKO's HercepTest™ (Glostrup, Denmark) (Figure 1 a-d). According to the scoring guidelines, the cases examined were classified as follows: Score 0: no staining or membrane staining in less than 10% of the tumour cells; Score 1+: weak, incomplete membrane staining in more than 10% of the tumor cells; Score 2+: weak or moderate complete membrane staining in

more than 10% of the tumor cells and 3+: strong, complete membrane staining in more than 10% of the tumor cells. Scores of 0 and 1+ were considered to be negative for HER2/neu expression while 2+ and 3+ were considered to be positive (overexpression).

Using a semi-automated system (hardware features: Intel Pentium IV, Matrox II Card Frame Grabber, Digital Camera Microwave Systems, ? icroscope Olympus BX-50; software features: Windows XP Pro/Image Pro Plus, version 3.0-Media Cybernetics 1997), areas of interest-including 0, 1+, 2+ and 3+ HER2/neu expression in PDAC tissues-were identified at a magnification of 40x and saved as high quality digital image files (JPGs at a resolution of 1,280x960). The complete digital image gallery consisted of 150 files corresponding to 50 sets (three images per case). Staining intensity values for HER2/neu overexpressed cases (evaluated as 2+ and/or 3+) were obtained by the implementation of a macro based on the DAB stain which was used as a chromogen substrate in the IHC protocol. A light to dark brown color range was considered acceptable for the measurements where the membranous stain was observed to be complete. Image analysis was based on an RGB 8-bit protocol with a range of 256 (0-255) continuous values (Figure 1f). All measurements were performed inside an active window of 16,848 μm^2 . All numerical data were filed on Microsoft Excel sheets.

Two pathologists (independent reviewers) screened and evaluated all the immunostained tissue microarrays spots of the slide according to the above-mentioned semi-quantitative scoring protocol and also all the digital image files (50 sets of images) without knowing the corresponding cases (Figure 1e). They used the same microscope at the same magnification (40x) and under the same technical conditions as had been used for the construction of a digital database (modification and standardization of the intensity index via the adjustment of parameters such as brightness, contrast, and gamma correction).

Chromogenic In Situ Hybridization (CISH) Assay

CISH SPOT-Light (Chromogenic ISH Detection Kit, Zymed, San Francisco, USA) was applied. The CISH polymer and the HRP Detection Kit (Zymed, San Francisco, USA) include steps similar to IHC. CISH assay for the detection of the HER2/neu gene and chromosome 17 status was performed on 5 µm thick serial, paraffin sections of the TMA block, adjacent to those used for IHC. Two slides were incubated at 37°C overnight followed by 2 hours incubation at 60°C. Then they were deparaffinized in xylene 2 times, 5 minutes each and in ethanol 3 times, 3 minutes each. The slides were incubated in Tris-EDTA buffer in a microwave oven at 700 W and at 95°C for 10 min. Those sections were treated enzymatically using pepsin digestion at 37°C for 5 min, washed in PBS and dehydrated in a graded series of ethanol and air dried. Twenty µL of ready-to-use digoxigenin-labeled HER2/neu probe and biotin-labeled chromosome 17 centromeric probe were applied to each TMA section, respectively. The tissue sections containing the added probe were denatured by placing the slides in a PCR machine equipped with a slide block at 95°C for 5 min. The slides were then placed in a moist slide box and incubated at 37°C for overnight hybridization. After hybridization process, the sections were carefully washed in 0.5x standard saline citrate at 75°C for 5 min. TMA sections were placed in 3% H₂O₂ and diluted with methanol for 10 min to block endogenous peroxidase. To block unspecific staining, Cas BlockTM (Zymed/InVitrogen, San Francisco, USA) was applied and incubated for 10 min. Following incubation with mouse anti-dig and then polymerised HRP conjugated anti-mouse for 45 min, the HER2/neu probe was visualized by DAB development (CISH Polymer Detection Kit, Zymed, San Francisco, USA) for 30 min. Similarly, the biotin-labeled Chr 17 centromere probe was detected by incubation with HRP-conjugated streptavidin for 30 min, followed by DAB development (CISH Centromere Detection Kit, Zymed, San

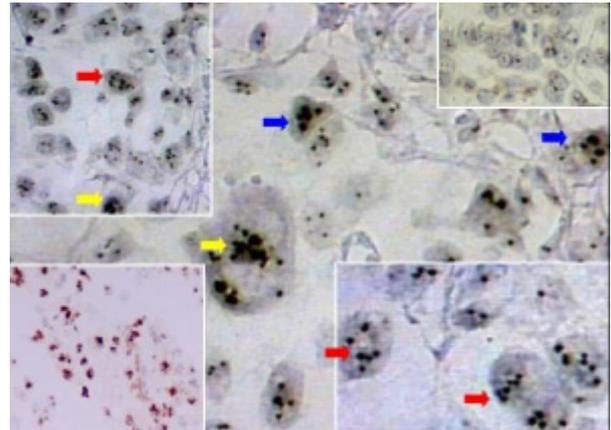


Figure 2. HER2/neu gene amplification detected by chromogenic in situ hybridization in pancreatic ductal adenocarcinoma. Note the different patterns of amplification: small (blue arrows) and large (yellow arrows) clusters of accumulated gene copies and also their scattered signals (red arrows). Upper right: normal (diploid) HER2/neu status in a case of pancreatic ductal adenocarcinoma; note two (predominantly) gene copies per nucleus. Lower left: HER2/neu high-level gene amplification in a case of breast adenocarcinoma; note small or large clusters of accumulated gene copies (original magnification: 40x).

Francisco, USA) for 30 minutes. Finally, TMA sections were lightly counterstained with hematoxylin and coverslipped with HistomountTM (Zymed/InVitrogen, San Francisco, USA). At the end of the process, CISH centromeric signals or gene copies were easily visualized as dark brown or blue, scattered or in small/large clusters dots, using a conventional, bright-field microscope at low magnification (10x, 20x, 40x) (Figure 2). Interpretation of HER2/neu gene and chromosome 17 centromere signals was based on Zymed's HER2 CISHTM Test Interpretation Guide. According to this, in cases characterized as high gene amplification, we observe more than 10 dots or large clusters of HER2/neu gene copies per nucleus in more than 50% of the cancer cell subpopulations examined. A low amplification level is characterized by the detection of 5 to 10 dots or small clusters of gene copies per nucleus but, in this case, chromosome 17 status has to be assessed for the identification of diploidy or chromosome polysomy. Finally, the presence of 3-5 dots of the gene is due to polysomy whereas 1-2 are

due to normal gene and chromosome status. Interpretation of the staining intensity values, and combined IHC and CISH results are described in Table 2.

ETHICS

The local ethical committee gave the Department of Pathology (417 VA Hospital-NIMTS, Athens, Greece) permission to use the tissues for research purposes. Oral informed consent was obtained from each patient and the study protocol conforms to the ethical guidelines of the "World Medical Association Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects" adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964, as revised in Tokyo 2004.

STATISTICS

Associations between variables including IHC (score and optical density levels), gene and chromosome status with clinicopathological parameters such as grade and stage were performed by the application of the chi square test (SPSS Inc Chicago IL v.11.0). Cohen's inter-rater kappa was also estimated along with its 95% CI to evaluate concordance

between the two methods used for the interpretation of HER2/neu IHC results. By its definition, a kappa value of 1 denotes complete agreement, values of more than 0.75 are characterized as excellent agreement, values between 0.40 and 0.75 show fair to good agreement, values more than 0 but less than 0.40 show poor agreement, and a kappa value of 0 indicates that the observed agreement is equal to chance. Two-tailed P values less than 0.05 were considered to be statistically significant.

RESULTS

CISH Analysis

CISH results were successfully obtained from all fifty PDAC cases. According to the CISH Test Interpretation Guide, HER2/neu gene amplification was detected in 8/50 cases (16%). In 3 of those amplified cases, more than 10 scattered dots and/or large clusters, representing scattered or accumulated gene copies, respectively, were detected in cancer cell subpopulations (high gene amplification status). In contrast, low gene amplification status was identified in 5 (10%) cases (6-10 scattered dots or small clusters). When correlating HER2/neu gene status to grade

Table 2. CISH and IHC correlations in 50 patients with pancreatic ductal adenocarcinoma.

| Variable | Grade | | | Stage | | |
|----------------------------------|-------------|-------------|-------------|------------|-------------|------------------|
| | 1 (n=10) | 2 (n=20) | 3 (n=20) | I (n=9) | II (n=5) | III-IV (n=36) |
| HER2/neu gene | | P=0.326 | | | P=0.582 | |
| Normal (n=42) | 7 (17%) | 17 (40%) | 18 (43%) | 7 (17%) | 5 (12%) | 30 (71%) |
| Amplification (n=8) ^a | 3 (38%) | 3 (38%) | 2 (25%) | 2 (25%) | 0 | 6 (75%) |
| Chromosome 17 | | P=0.682 | | | P=0.370 | |
| Normal (n=31) | 5 (16%) | 13 (42%) | 13 (42%) | 7 (23%) | 2 (6%) | 22 (71%) |
| Aneuploidy (n=19) | 5 (26%) | 7 (37%) | 7 (37%) | 2 (11%) | 3 (16%) | 14 (74%) |
| HER2/neu protein | | P=0.019 | | | P=0.466 | |
| 0 (n=29) | 9 (31%) | 12 (42%) | 8 (27%) | 4 (14%) | 2 (7%) | 23 (79%) |
| 1+ (n=11) | 1 (9%) | 6 (54%) | 4 (37%) | 3 (28%) | 2 (18%) | 6 (54%) |
| 2+ (M; n=6) ^b | 0 | 2 (33%) | 4 (67%) | 2 (33%) | 0 | 4 (67%) |
| 3+ (H; n=4) ^b | 0 | 0 | 4 (100%) | 0 | 2 (50%) | 2 (50%) |

^a Three high and 5 low gene amplification levels

^b Moderate (M) and high (H) staining intensity values (estimated cut offs: 118-139, and 0-110, respectively). Values range between 0 (black) and 255 (white) demonstrating strong expression (values decreasing to 0) and loss of expression (values increasing to 255).

and stage of the tumours examined, we failed to observe statistical significance (P=0.326 and P=0.582, respectively). Chromosome 17 analysis detected aneuploidy (3-5 centromeric signals per nucleus) in 19 cases (38%). Two of them were associated with high gene amplification cases. In all other cases demonstrating chromosome 17 aneuploidy, we observed the same mean number of HER2/neu signals (3-5 signals per nucleus) whereas, in the rest of the cases characterized as normal (diploid) regarding the specific chromosome and gene status, we primarily detected two centromeric and gene copies per nucleus. Similarly, statistical significance was not reached by correlating chromosome 17 status and clinicopathological parameters (P=0.370) for stage, and P=0.682 for grade) or HER2/neu gene and chromosome 17 status (P=0.354)

IHC Analysis

IHC results were also successfully obtained from all fifty PDAC cases. Both of the pathologists (independent reviewers) evaluated 29/50 cases (58%) as 0. In contrast, when evaluating the other 21/50 cases (42%), they had controversial results, characterized by discordance. But, when they evaluated the same fifty sets of images obtained by digital microscopy, a significant improvement in interobserver agreement was reached (0 score: 100%; 1+: 100%; and 2+/3+ group: 100%) although, in the group of overexpression (2+ and 3+), borderline differences of estimation were again present (concordance between 80-84%). Similarly, each of them demonstrated improvement when comparing his/her results

obtained using the two methods. A significant degree of increased concordance was observed when comparing kappa values (0.76 and 0.94, for conventional and digital microscopy regarding 2+ and 3+ groups, respectively) as described in Table 3.

Evaluation of 10/50 (20%) cases which were characterized as 2+ or 3+ by both of the reviewers based on the digital image gallery was also performed by computerized image analysis. High levels of staining density in HER2/neu complete membranous expression were detected in 4 cases whereas the other 6 cases demonstrated medium levels, according to the Staining Density Interpretation Guide, as modified for the purposes of the current study. Based on the digital microscopy results which demonstrated absolute concordance with the computerized image analysis regarding the main groups of HER2/neu expression classification (0, 1+ and 2+, 3+), a significant statistical association was observed correlating them to grade (P=0.019), but not to stage (P=0.466). Finally, a significant statistical association was observed correlating overall protein expression and gene status (P=0.006). Interpretation of the combined IHC and CISH results showed that not only the majority of the overexpressed protein cases (2+, 3+) demonstrated gene amplification, but also some cases characterized as 1+ (n=2).

DISCUSSION

In the present study, we evaluated the levels of HER2/neu protein expression in fifty cases of PDAC based on TMAs, using two methods of IHC interpretation. Conventional eye

Table 3. Immunohistochemistry results based on digital microscopy.

| CISH | IHC | | | | | |
|----------------------------|--------------------------|----------|----------|--------------------------|----------|----------|
| | 1 st Reviewer | | | 2 nd Reviewer | | |
| | 0/1+ | 2+ | 3+ | 0/1+ | 2+ | 3+ |
| Nonamplified (n=42) | 38 | 2 | 2 | 38 | 3 | 1 |
| Amplified (n=8) | 2 | 3 | 3 | 2 | 3 | 3 |
| Total (n=50) | 40 | 5 | 5 | 40 | 6 | 4 |

Kappa values (and 95% confidence intervals) between 1st Reviewer and 2nd Reviewer:

- digital microscopy (overall): 0.96 (0.93 to 0.99)

- digital microscopy (2+, 3+): 0.94 (0.88 to 0.99)

microscopy performed by two pathologists showed that, in some cases characterized as immunohistochemical overexpression (2+, 3+), there were different estimations. Using the same Interpretation Guide those independent reviewers failed to completely agree when evaluating the cases of overexpression. Although their results demonstrated high concordance in groups 0 and 1+, in cases characterized as 2+ and 3+, a medium degree of discordance was observed. In contrast, evaluation of the cases based on a digital image gallery including high-resolution files remarkably improved the level of concordance. Furthermore, computerized image analysis was performed in order to discriminate between the 2+ and 3+ subgroups, according to the level of staining density (high-medium values). Those cases were eligible for analysis and not the cases of 1+ or 0 because by definition the critical point in digital image analysis concerning HER2/neu protein expression is the presence of a complete (entire) "ring like" membranous stain.

During digital analysis procedure, those cases which were characterized as borderline by the two reviewers were found to be distinct (different numerical values). This observation is easily explained because the human eye can distinguish less than 200 grey levels of optical density. In contrast, commercial available image analysis software based on 8-bit or higher processors discriminate at least 256 continuous intensity values [16, 17]. Furthermore, the reviewers had to evaluate HER2/neu expression based on a protocol characterized by the co-estimation of two parameters: membrane pattern (complete or incomplete, homogeneous or heterogeneous) and level of overexpression (light to dark brown stain). We feel that every reviewer over- or under-estimates one of those two variables in borderline cases, and this subjective estimation influences the final interpretation. This observation reflects the limited ability in discriminating those borderline cases performed by conventional eye microscopy.

Two recently published studies investigated the potential usefulness of digital image microscopy in the interpretation of HER2/neu expression on breast cancer specimens [18, 19]. The authors concluded that the assistance of digital microscopy improves the accuracy and reliability of immunohistochemical estimation. In fact, significant improvement concerning overall concordance of those independent reviewers was achieved when they evaluated the same high-resolution digital images.

Reviewing the methodologies of those studies, we have to observe that computer-assisted image analysis is a powerful tool for the evaluation of immunohistochemical stains, such as HER2/neu. Image-based computerized analytical procedures in pathology and cytology are characterized by increased accuracy, are easy to use and have decreased costs [20]. New software including improved tools for digital analysis allows for more complicated applications. Although segmentation, which means delimitation of the boundaries between two compartments, remains an obstacle in the field of technical improvement, morphometric data obtained by the design and application of novel macros increase the levels of accuracy concerning immunohistochemical interpretation [21, 22]. In the current study, the performance of digital microscopy and the additional evaluation of staining intensity levels increased the accuracy in interpreting the HER2/neu expression, decreasing or eliminating differences originating from conventional eye microscopy. Focusing on the comparison between the HER2/neu gene and protein alterations in breast cancer and PDAC we observe that although this approach seems to improve the assessment of protein expression in breast cancer, gaining valuable information about HER2/neu gene status, there is no evidence that it affects the clinical significance in PDAC. In breast cancer, HER2/neu gene amplification is strongly correlated to protein overexpression (predominantly 3+), whereas in PDAC not only 3+, but also 2+, even 1+ cases of protein

expression demonstrate this gene deregulation mechanism. We can speculate that, not only gene alterations, such as amplification (double minutes type), but also other mechanisms of deregulation (i.e., intragenic mutations) are associated with HER2/neu overexpression in PDAC, involving chromosome 17 instability, similar to breast cancer [6].

Simultaneous strong protein expression and gene amplification characterize a minority of PDAC cases as compared to breast cancer, but this subset appears to be eligible for targeted therapy. It has recently been shown that trastuzumab inhibits the growth of positive pancreatic carcinoma cell lines *in vitro* and *in vivo* [23]. Furthermore, alterations of the molecule have already been assessed in other types of cancer, such as the lung or stomach, but their biological significance and predictive value is under investigation [24, 25]. When analysing gene and protein levels based on tissue microarrays, those two studies showed that in patients with non small cell lung carcinoma demonstrating simultaneous HER-2/neu gene amplification and strong protein expression, there is a pronounced tendency for shorter survival, that amplification is common in the intestinal type of gastric carcinoma and that it is also correlated with a poor outcome.

In this study, we also performed a CISH protocol in order to evaluate the HER2/neu gene and chromosome 17 status. IHC analysis is characterized by the inability to identify the specific molecular deregulation mechanism which is responsible for the protein overexpression. For many years, IHC has been the main method for evaluating HER2/neu or other receptors, such as EGFR protein expression in pathologic samples [26, 27]. Despite its relatively low cost and straightforward concept, immunostaining results can be divergent, mainly due to the varying sensitivity and specificity of commercially available antibodies, differences in tissue processing, lack of a universal standard and interobserver differences in evaluating the staining results due to subjectiveness [28]. Concerning

HER2/neu protein expression, there are many antibodies (monoclonal or polyclonal) which recognize different domains of the molecule. For example, clones, such as CB11 or Pab1, detect the intracellular part, the HerceptTest™ detects the membrane-intracellular part, whereas CBE1 or TAB 250 reacts with the extracellular domain of the receptor [29, 30]. Therefore, the interpretation of IHC analysis depends on the selection of the specific antigen epitope targeted by the corresponding antibody. Co-evaluation of the gene status with protein overexpression provides greater insight and meaningful information than protein overexpression alone. Recently, CISH has drawn more attention, since it is capable of evaluating gene amplification/deletion, chromosome aneuploidy or chromosomal translocations simultaneously with tissue morphology on the same slide, using routine light microscopy. This way, large regions of each tissue section can be scanned rapidly with hematoxylin counterstain. Several studies have shown that CISH is an alternative to fluorescence *in situ* hybridization (FISH) and that they both demonstrate a high level of concordance when their results (92-98%) are compared [31]. Because the novel targeted therapeutic strategies via monoclonal antibodies are designed on the basis of reaction with the extracellular domain of the specific receptor, the crucial process is the co-evaluation of IHC analysis and gene analysis (FISH, CISH, PCR) for the confirmation of gene amplification in many cancers [32].

We have already mentioned that HER2/neu gene amplification appears to be a very important prognostic factor for patients suffering from breast cancer because it correlates to the chemotherapy response (trastuzumab and anthracyclines) but, in PDAC cases, there are controversial data regarding its biological significance [33, 34, 35]. In our study, gene and chromosome 17 status were not correlated to biological parameters, including grade and stage, whereas HER2/neu expression was found to be associated only with grade. According to some studies using predominantly rabbit

antihuman HER2/neu polyclonal antibody (Hercept Test or Pab 1) which targets the membrane-intracellular domain, there are conflicting results about protein over-expression [36, 37].

In conclusion, this study suggests that, although significant biochemical and technical progress has been achieved in the field of immunohistochemistry, interpretation of the results is characterized by subjectiveness. Concerning PDAC, identification of the gene amplification combined with strong protein expression may provide a rational way for selecting patients who are potentially eligible for targeted therapeutic approaches via monoclonal antibodies. Computer-assisted image analysis also represents a modern method characterized by increased accuracy and improved reliability regarding the interpretation of HER2/neu protein expression in solid tumors, such as breast or pancreatic cancer.

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Keywords Carcinoma, Pancreatic Ductal; Genes, erbB-2; Gene Amplification; Immunohistochemistry; In Situ Hybridization

Abbreviations CISH: chromogenic in situ hybridization; FISH: fluorescence in situ hybridization; IHC: immunohistochemistry, PDAC: pancreatic ductal adenocarcinoma; TMA: tissue microarrays

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