Author’s response to reviews

Title: Quantitative real-time RT-PCR and chromogenic in situ hybridization: precise methods to detect HER-2 status in breast carcinoma.

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Author’s response to reviews: see over
October 29, 2008

Dr. Scott Edmunds  
Senior Editor  
BMC Cancer

Ref. MS:9787917272052548

Dear Dr Scott

We would like to thank the editors and the reviewers for their constructive criticisms regarding our manuscript, entitled: “Quantitative real-time RT-PCR and chromogenic in situ hybridization: precise methods to detect HER-2 status in breast carcinoma” by Fabíola Encinas Rosa et al. The comments were very helpful and have led us to make several changes in the manuscript. According to the suggestions, we included additional information corresponding exclusively to the subject of this research in a concise manner.

We append each point the reviewers made and the alterations are highlighted in gray. We have indicated the page and paragraph numbers in which changes or additional information has been included in the revised manuscript. All authors have read and approved the revised version of this manuscript.

We look forward to hearing from you concerning the suitability of the revised manuscript for publication.

Sincerely,

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OVERALL RESPONSE TO GENERAL COMMENT CONCERNING STUDY PURPOSE

EDITOR

1 - We recommend that you ask a native English speaking to help you copyedit the paper. The manuscript was revised by a British-born scientific text editor.

2 - Experimental research that is reported in the manuscript must have been performed with the approval of an appropriate ethics committee. Could you clarify which committee granted it? Informed consent must also be documented. The Ethics Committee from Amaral Carvalho Hospital Foundation approved this study (CEPFHAC 007/05) and this information is described in Material and Methods section (page 6, 1st paragraph). The patients were informed of the research and all signed a permission form allowing us to analyze samples that were normally collected during the surgery, causing no additional discomfort to them. The individual Consent Form is available at the Senology Department, from Amaral Carvalho Cancer Hospital, Jau, SP, Brazil. Dr Jose Roberto Figaro Caldeira (caldeira@netsite.com.br), co-author of this manuscript, and the surgeon responsible for these consents. We attached the official document approving the research and the Consent Form used for this study.

3 - Please include a 'Competing interests' section between the Conclusions and Authors' contributions. We included the following paragraph in the appropriate section: “The authors declare that they have no competing interests” (page 21).

5 - Financial competing interests
There are no financial competing interests.
1 - Major Compulsory Revisions

1.1 - The authors compare IHC, CISH and qRT-PCR but do not include FISH. They state that FISH is difficult in use and that the accuracy of this technique is questionable. In fact, FISH has been proven to be one of the most accurate techniques currently available due to the use of fluorescence. Before drawing conclusions on the use of CISH and/or qRT-PCR for HER-2 testing, they should at least compare these methods to FISH analysis.

The reviewer is correct to point out that FISH is an accurate methodology and currently used by HER-2 status in breast carcinomas. We excluded the contradictory comments regarding FISH in the Abstract and Background sections.

As suggested by the reviewer, we compared CISH methods with FISH analysis in 11 cases (Figure 1, letter). We performed this analysis in 8/13 cases presenting high-level amplification, because just these histological sections were available. Unfortunately, the paraffin blocks were exhausted in previous studies (Caldeira et al. *BMC Cancer* 6:48, 2006; Rosa et al. *Hum Pathol* 39:720, 2008) or they form part of the Hospital’s bank of samples exclusively devoted to diagnosis. The FISH analysis description and detailed results are presented in the manuscript in the appropriate sections (highlighted in gray).

1.2 - IHC is performed using the relatively new “rabbit monoclonal” antibodies...However, it is known that these antibodies tend to produce false positive results due to their extremely high sensitivity. The authors should first validate this assay against for instance HercepTest before drawing conclusions. Besides, there is a relatively high number of cases negative on IHC (score 0/1+) but positive on CISH/RT-PCR.

The SP3 antibody (rabbit monoclonal) that we used in this study is a new line of antibodies with higher sensitivity and specificity than monoclonal traditional antibodies from mouse. Recently, an article was published comparing HercepTest with SP3 by immunohistochemistry (Wludarski and Bacchi, *Appl Immunohistochem Mol Morphol* 16:466, 2008). The goal of this study was to evaluate the concordance, sensitivity and specificity of SP3 compared to fluorescence in situ hybridization and HercepTest in 179 invasive breast carcinomas. The authors found that HercepTest presented higher sensitivity (100% vs. 89%); however, SP3 showed higher specificity (97% vs. 89%). An important advantage of SP3, in comparison with HercepTest, is its greater discrimination power (72.1% vs. 34.1%, respectively). The authors concluded that SP3 antibody could be helpful in the determination of HER2 status on a routine basis. In our study, 15% and 20% of cases were negative for IHC (score 0/1+) but positive for CISH/qRT-PCR. However, our study demonstrated that CISH and qRT-PCR showed a high level of concordance. Using a similar comparison, Wludarski and Bacchi (2008) detected that 4.3% (4/93) of cases scored as 0/1+ by SP3 presented amplification by FISH. These data
suggested that the discrepancies are not related to the antibody used in the present study and that HercepTest and SP3 are comparable in efficiency for evaluating HER-2 status by immunohistochemistry.

1.3 - CISH is used without internal control for the centromere of chromosome 17. As a consequence, tumors showing a modest increase in HER-2 copy number due to polysomy 17 will be considered "low-level amplified" by CISH. The authors did not seem to take this (frequent!) phenomenon into account and need to complete their results with dual-colour FISH or CISH analysis.

We are unsure as to what the reviewer refers to here but we believe that she is questioning the absence of cases with “low-level of amplification”. Using CISH, we found 24 out of 37 cases presenting nonamplification (those tumor cells with two to five brown intranuclear spots per nucleus) and 13 out of 37 cases with high-level amplification (defined as more than 10 copies per nucleus or when copy clusters were observed in more than 50% of cancer cells). In fact, three cases (Figure 2) presented mixed area with cells showing “low-level of amplification” (7.5% of cases). Several authors have described a similar frequency of cases showing low-level of amplification by CISH (6%, 10.6%, and 10.6%) (Li-Ning et al. Int J Surg Pathol 13:343, 2005; Madrid et al. Breast Cancer Res 6:R593, 2004; Sartelet et al. J Clin Pathol 58:864, 2005; respectively). We evaluated 11 cases by dual color FISH: two cases scored as 2+ by IHQ; six cases scored as 3+ by IHQ, and the three cases presented in Figure 2 (those three cases that presented low-level of amplification cells) (Figure 1, letter). The results and their comparisons are included in the manuscript in the appropriate sections (highlighted in gray).

2. Minor essential revisions
2.1 - The authors should shorten the Materials and Methods section in general. Is the first paragraph of the results section a repetition/summary of the materials and methods section? More concise writing would improve the manuscript substantially.

The Introduction and Material and Methods sections were summarized, as suggested by the reviewer. The first paragraph of the Results section is a summary of the clinical data and was included in the Material and Methods section (page 6, 2nd paragraph) because we believe that this information will be useful to clinical readers of BMC Cancer.

2.2 - The authors should explain more clearly how they performed microdissection. To achieve 90% pure invasive tumor component and successfully extract RNA for all cases is remarkable...

Although two reviewers suggested shortening of the manuscript, three of them raise questions involving the microdissection procedure. We have included the procedure in the Material and Methods section (page 8,3rd paragraph).
2.3 - Why did the authors perform CISH on only 43 samples and IHC on 75? Are the paraffin sections used for IHC/CISH consecutive? Was the intratumor heterogeneity on DNA level also present on protein level?

As described above, all cases of the present study were evaluated previously and resulted in two published manuscripts (Caldeira et al. *BMC Cancer* 6:48, 2006; Rosa et al. *Hum Pathol* 39:720, 2008). Unfortunately we were unable to complete the CISH analysis in all cases as we would have preferred. The slides obtained from paraffin sections used for this study were consecutive for CISH and IHC procedures. In 8 out 11 cases also evaluated by FISH (Figure 1, letter), the slides were consecutive (serial). In three of them we used the last slides available.

We believe that the question involving the intratumoral heterogeneity is related to the Figure 2. In IHC and CISH/FISH procedures, the slides were randomly distributed to independent blinded observers. Any discrepancies between sample classifications were addressed by immediate review and the final result was reached by consensus. The DNA heterogeneity was observed in only three cases by CISH.

3 - Quality of written English: Needs some language corrections before being published;

The manuscript was revised by a British-born scientific text editor.
REVIEWER 2 – Luciane Regina Cavalli

MINOR ESSENTIAL REVISIONS

Introduction section:

1 - The name of the gene should be capitalized and in italic: HUMAN EPIDERMAL GROWTH FACTOR RECEPTOR TYPE 2 (Page 3-2nd paragraph)

The official symbol of the ERBB2 gene must be in italic and capitalized. The official name is “v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)”. The symbols of the genes are included on page 4, 2nd paragraph, as suggested by the reviewer.

2 - There are some misconceptions about the FISH and qRT-PCR methods (Page 4-1st paragraph). It is without question that there are significant advantages of CISH over FISH analysis for the detection of gene copy number changes. One of them includes cost effectiveness and the practicality. Both of the methods, and not only CISH, can preserve the morphological characteristics of the tissue section, allowing a simultaneous genotype-phenotype correlation. Although qRT-PCR can be less subjective than the CISH/FISH or IHC score analysis, it is not insensitive to inter-observer variability. In fact in the discussion section the authors themselves point out this subjectivity issue (Page 16 - 2nd paragraph). The analysis of this method is also affected by the way the data evaluation is conducted, and if performed inappropriately, can highly affect the quality and reproducibility of the results (See refs: Bustin et al, Journal of Biomolecular Techniques, v.15, n.3, 2004; Nolan et al, Nature Protocols, v.1, n.3, 2006).

The CISH procedure has some advantages over FISH. One of these is the fact that CISH permits the concurrent analysis of morphological features of the tumor/cells and gene copy numbers, making the identification of the components of interest easier (for example, differentiation between high grade invasive ductal carcinoma with comedo-like growth pattern and comedo-like ductal carcinoma in situ) or distinguishing (Lambros et al. Hum Pathol 38:1105, 2007). In addition, in FISH analysis, tissue morphology and gene amplification are primarily disconnected because the tumor cells for signal evaluation are based on nuclear DAPI or propidium iodide staining, which does not always permit adequate histopathological evaluation of the cells (Hauser-Kronberger and Dandachi, J Molec Histol 35:647, 2004). In fact, the FISH slides have to be viewed in conjunction with a standard hematoxylin-eosin section from the same block that will enable morphological correlations and identification of the invasive tumor (Hicks and Tubbs, Hum Pathol 36:250, 2005).

The sentence describing CISH and FISH was altered, as suggested by the reviewer (Abstract and Background sections). We included a paragraph describing the qRT-PCR limitations (Background section, page 4, last paragraph).
3. There is no general reference for the qRT-PCR method. It should be included, since it is relevant for the readers that are not familiar with the method (Page 4, 1st paragraph). As suggested, we included the reference Livak et al. (2001) in “Real time quantitative RT-PCR (qRT-PCR)” section (page 9, last paragraph).

Methods section:

1. In the analysis of the CISH and IHC slides, the scoring was performed by three and two independent observers, respectively. Therefore it would be interesting to provide the inter-observer agreement values (e.g. for two observers: the inter-observer variability value can be assessed as follows: the joint rating of the two observers can be displayed on a 2x2 contingency table, and the level of agreement between them can be assessed by Kappa statistics) (Page 6, last paragraph and page 9, 1st paragraph).

For CISH analysis, a concordance of 100% occurred between the two observers for 37 cases. The three additional cases presented in Figure 2 of the manuscript were described in details and analyzed by three observers. They were excluded from the statistical analysis because we found areas with different HER-2 hybridization signals by CISH. We detected nonamplified HER-2 gene (2-5 copies per nucleus), low-level amplification (6-10 copies or small clusters) and high-level amplification (>10 copies or large clusters) in different areas of the same tumor. For this reason, we believed that the Kappa test is not applicable in our study. These data presented in the manuscript were obtained by consensus among the observers, as described in the appropriate sections. We observed no disagreement between FISH and CISH results. Besides, all observers obtained a consensus of the results by IHC analysis.

2. There is no reference for the scoring system used for HER-2 CISH analysis. The authors should cite the reference (Page 7, 1st paragraph).

HER-2 CISH analysis was performed according to the manufacturer’s instructions (Zymed SPoT-Light HER2 CISH Kit), which was included in the “HER-2 copy number alterations” section (page 7, last paragraph).

3. How were the frozen samples microdissected prior to the RNA isolation? (Page 5, 2nd paragraph).

The microdissection procedure details were included in Material and Methods section on page 8, 3rd paragraph, as suggested by the reviewer.

4. The authors should describe in the qRT-PCR method, the primer and probes sequences used for the HER-2 analysis. Considering the wide variability in primers and probe designs, this information is
relevant and can present implications for the reproducibility of the data and its equivalent comparison from the ones in the literature (Page 8, 2nd paragraph). In fact the authors indirectly mention in the discussion section (Page 16, 2nd paragraph) the consequences of this variability (differences in melting temperature, and in genomic vicinity of the amplicon) in the data analysis.

The primers and probes were made by Taqman Gene Expression Assay from Applied Biosystems (assays Hs00170433_m1 and 4326317E). We included the assays number in the Material and Methods section (page 9, 1st paragraph).

5. I would suggest that the authors describe and define Ct for the readers that are not familiar with the qRT-PCR method analysis (Page 8, 2nd paragraph).

The Ct (Cycle threshold) is defined as the cycle when sample fluorescence exceeds a “chosen” threshold above the calculated background fluorescence. The word “chosen” is used since the fluorescence is not a constant or absolute value, but is influenced by changing reaction conditions (Bustin and Nolan, J Biomolec Tech 15:155, 2004). Two reviewers suggested substantially shortening the manuscript. Moreover, we believe that this information is very well established in molecular assays and available in many technical manuscripts in the literature.

Results section:

1 - The results are not presented in a very clear manner. I believe it can be improved by dividing the results into sections: CISH analysis, IHC analysis and qRT-PCR analysis, prior to the comparison of the methods (Pages 10 and 11). A title prior to the comparison with the clinico-pathological data should be included (Page 12, last paragraph).

As suggested by the reviewer, we included a subtitle “Clinico-pathological data in comparison with HER-2 status” in the Results section (page 14, 3rd paragraph).

2 - There is information related to patients follow-up in the first paragraph of this section (page 10), however no correlation results were presented. Were they not tested or they were negative?

These data are relevant and for this reason they are included in the manuscript. However, disease-free survival and overall survival (OS) curves calculated by the Kaplan-Meier method, and log-rank tests were not performed because the number of cases was limited. During the interval of the study, five patients died due to unrelated causes, two died of the disease, and six missed their follow-ups.

3 - The description of the results of the three cases that present heterogeneity in relation to CISH analysis is confusing. The areas should be described by numbers; i.e. area 1 and area 2. (See revision for Figure 2) (Page 10-last paragraph and page 11st paragraph).

The reviewer is correct to point out that the different areas from Figure 2 should be presented. We included these suggested alterations in the text (page 12, 2nd paragraph) and in Figure 2.
Discussion section:

1 - No general reference for FISH analysis for the HER-2 gene is cited (Page 13 – 1st paragraph of this section).

We altered this sentence and included the reference Hicks and Tubbs (2005), on page 16, 1st paragraph (reference 19).

2 - The meaning of the sentence “According to Wolf et al … as a predictor of benefit from anti-HER 2 therapy” is not clear. It should be re-written (Page 13, last sentence).

The sentence was rewritten as suggested by two reviewers (page 16, 1st paragraph).

3 - Based on the literature the practicality of applying qRT-PCR for HER-2 transcript analysis in the clinical setting is still not clear. There are several aspects that require critical consideration, including: the standardization of the qRT-PCR protocols, consistency with regards to reagents used, consideration of the assay design, and the analytical methods. An interesting “guideline” containing useful considerations of the several aspects of the procedure, from the RNA isolation to the reverse transcription step and PCR amplification are described in detail by Nolan et al, 2006 (Nature Protocols v.1, n.3). The authors should discuss this.

In the present study, we followed all the recommendations suggested by Nolan et al. (2006). In summary, the RNA quality was assessed, the cDNA synthesis was developed according to a single method, cDNA priming method was specific, together with constant timing and reaction temperature, normalization was transparent and internal reference genes were validated for each experimental set-up, amplification of target-specific standard curves was analyzed, any signal detected in the negative controls was reported and qualified by melt curve, and when reporting data as a relative change (i.e., when using DDCt analysis), the Ct range of target detection was quoted. The qRT-PCR procedures are completely standardized in our laboratory (recent articles published: Rosa et al. Hum Pathol 39:720, 2008, Silveira et al. Leuk Res, 2008, Epub ahead of print). Since two reviewers suggested substantially shortening the manuscript, we eventually decided not to include these considerations.

4 - In the beginning of the discussion the authors should include a few sentences related to the objective of their study, before directly discussing the data (Page 14, 2nd paragraph).

We sympathize with the reviewer’s concern but also feel that the purpose of the study was presented in the Introduction section. As explained above, we agree with the reviewers regarding shortening the manuscript.

5 - “The melting temperature of the amplicon and the behaviour of the genomic vicinity of the amplicon, especially in the first PCR cycles can affect the qRT-PCR interpretation”; this is not a
subjective issue and it is not related to intra-observer error. The way the sentence is written is confusing and should be clarified (Page 16, 2nd paragraph).
We modified the sentence according to the suggestion (page 19, 2nd paragraph).

Figures:
Figure 2: The different tumor areas (represented by the graphic pies) analyzed for each case (A, B and C) should be labeled. The legend of the figure should be corrected accordingly.
We altered the legend and the Figure as suggested. We have included the FISH results of the same cases in the Figure 2.

Figure 3: Figure 3C is out of place. It is cited in the text after figure 4 (Page 12- 3rd paragraph) and it should not be included together with Figures 3A and 3B, which illustrates comparison among the methods. Therefore, figure 3C should be replaced by figure 4. The legend of the figure should be corrected accordingly. Figure 4: Should be replaced by Figure 3C. This figure illustrates correlation with qRT-PCR and a clinico-pathological characteristic (lymph node status) of the patients. This should be presented separately and the legend should be corrected accordingly.
The suggestion was accepted, Figure 4 was replaced by 3C and the legends were corrected.

Language:
There are several observations concerning the English language, which require the author’s attention. These include grammatical errors and spelling. A revision of the language should be performed.
The manuscript was revised by a British-born scientific text editor.

DISCRETIONARY REVISIONS
Material and methods section:
1 - The CISH and IHC analysis for HER-2 using the Zymed and DAKO protocol, respectively, are well known and widely used protocols. The authors could therefore have described both in a summarized manner.
The CISH and IHC analysis were summarized as suggested by the reviewer.

Tables: Table 1. In the table description the methods should be listed according to the order that they are presented in the table, i.e. CISH, qRT-PCR and IHC.
We agree with the reviewer and altered the order of the methodologies in the table description.

Quality of written English: Needs some language corrections before being published;
The manuscript was revised by a British-born scientific text editor.
Discretionary Revisions

1 - Some limitations of this work like the use of fresh frozen tissues for real time RT-PCR is not clearly stated. More specifically, the authors state in their final conclusion paragraph that qRT-PCR is fast, reliable and semi automated and can be performed in most pathology laboratories. There is no doubt about that, however the authors have also to state that real time RT-PCR for HER-2 mRNA requires fresh frozen tissue samples.

We thank the reviewer for the clarification and we included this explanation in the conclusion of the Discussion section (page 21, 1st paragraph).

Minor Essential Revisions

1 - In the Materials and Methods section the authors should give a Table presenting the primer sequences used for the real time RT-PCR experiments for HER-2 and GAPDH.

The primers and probes were made by Taqman Gene Expression Assay from Applied Biosystems (assays Hs00170433_m1 and 4326317E). We included the assays number in the Material and Methods section (page 9, 1st paragraph).

Major Compulsory Revisions

1 - On page 15 the authors cite the study of Ntoulia et al (ref 22), when referring to HER-2 mRNA status as evaluated by real time PCR. However in this study HER-2 status was evaluated by real time PCR at the gene copy number level, and not at the mRNA level. On page 16 the authors also state that “gene expression median was correlated with gene copy number, a finding also observed by Ntoulia et al (22)”. This statement is correct.

We thank the reviewer for giving us the chance to correct this misunderstanding. We made the appropriate alterations as indicated.

2 - The authors should also give experimental data on the sensitivity of the real time PCR assay. What is the minimum number of HER-2 positive (over-expressing) cells that real time PCR can detect in the presence of normal cells that do not over-express HER-2? This experiment could be performed by dilutions of a known number of HER-2 over-expressing cancer cells (cell line) in a constant number of normal mammary cells. Otherwise the authors could cite similar studies on real time RT-PCR sensitivity for HER-2 mRNA.

We believe that this idea is very interesting; however, in the present study we used relative quantification to evaluate the transcript level of HER-2 in comparison to normal breast tissue as described in the Material and Methods section. However, we included in the Discussion section (page 19, last paragraph) a paragraph regarding the qRT-PCR sensibility: “Using PCR-based methods, the
expression of tumor- or tissue-specific genes and the presence of genetic abnormalities can be detected in a clinical specimen with higher sensitivity (one malignant cell out of $10^6$-$10^7$ normal cells) than that of other techniques such as light microscopy (one malignant cell out of $10^2$-$10^3$ normal cells). Using RT-PCR the nucleic acid molecules can be amplified $10^{10}$-fold (for review Mocelli et al., 2006). Moreover, HER-2 overexpression can be detected by qRT-PCR in 0.1 cell equivalent spiked into 8mL of peripheral blood and the detection limited increased to 10 and 50 cell equivalent per 8mL in cell lines expressing HER-2 intermediate and low levels (You et al., 2008).”
REVIEWER 4 – Silvana DiPalma

Major Compulsory Revision

1 - The paper should be shortened and more focused on the title. Most parts are not relevant to the study. The authors should focus on the relationship between HER2 gene status as detected by CISH, HER2 mRNA expression as detected by qRT-PCR and HER2 protein over-expression as detected by immunohistochemistry. These data could be described in 1/3 of the length of the current paper.

The paper was shortened as suggested by the reviewer. However, we have to include additional information as requested by the other three referees.

Suggested amendments (both major and minor):

1 - Abstract (p2)/ Background (p3-4). The sentence: ‘Considerable controversy exists regarding the relative accuracy of these methods’ is repeated in the background (p3-4) with no qualifying sentences. This should be expanded in the background section with references to back it up as this is rather a bald statement.

We are grateful for the opportunity to expand the Abstract and Background sections. We included the alterations as suggested by the reviewer (Abstract: page 2, 1st paragraph; Background: page 4, last paragraph).

2 - Background p3, 3rd paragraph. The sentence beginning ‘Reliable laboratory data in evaluating Her-2 status…….’ has a typo and should end either ‘avoids potential cardiotoxic effects in women not presenting with amplification and overexpression’, or ‘avoids potential cardiotoxic effects in women not exhibiting/showing amplification and overexpression’.

We altered the sentence as suggested (page 4, last paragraph).

3 - Materials and Methods: Chromagenic In Situ Hybridisation (CISH) (p6). Why were only 43 out of the 75 cases assessed using CISH? If all cases were assessed using all 3 methods this would potentially improve the paper by using larger numbers which would improve statistical relevance. If this work is not carried out, then reasoning behind using only 43 cases in the CISH study should be mentioned.

We agree with the reviewer that it would be very interesting to assess all the cases using the three methods. However, the CISH analysis was performed in a subgroup of breast cancer samples containing available material and, unfortunately, we were unable to complete the analysis in all cases, as we would have preferred. Unfortunately the paraffin blocks were exhausted in previous studies (Caldeira et al. BMC Cancer 6:48, 2006; Rosa et al. Hum Pathol 39;720, 2008) or they form part of the Hospital’s bank of samples exclusively devoted to diagnosis. We have included this information in the Material and Methods section (page 7, last paragraph).
4 - Materials and Methods: Total RNA isolation and reverse transcription (p7). This should mention that laser microdissection was used prior to this procedure as it is mentioned in the results only (p10, paragraph 2).
As requested by referees 1 and 2, we included the microdissection assay in the Material and Methods section (page 8, 3rd paragraph).

5 - Materials and Methods: qRTPCR (p8). Any positive controls used should be mentioned. Also the number of replicates should be stated.
We used four samples from normal breast tissue obtained from patients who underwent mammary reduction as reference. These cases were histopathologically confirmed as normal tissue. All of them were microdissected. In addition, we have standardized the protocol using breast cancer cell lines that overexpress HER-2 (HCC1419, AU565, and SKBR3 obtained from the American Type Culture Collection). The number of replicates (two) was included on page 9, last paragraph.

6 - Results (p12, paragraph 3). It is not uncommon to find that IHC2+ cases are negative by CISH.
We agree with this statement. In the section “CISH compared to IHC analysis” we reported the data obtained in the study.

7 - Discussion (p13). The sentence ‘The limitation of this procedure is the use of fluorescence microscopy and its implications technical’ should end ‘technical implications’. These technical implications should be described.
We excluded this sentence and included a new paragraph detailing the advantages of CISH in comparison to FISH (Introduction section: page 4, last paragraph; and Discussion section: page 16, 1st paragraph).
As we responded above (Reviewer 2), the CISH procedure has some advantages over FISH. One of these is the fact that CISH permits the concurrent analysis of morphological features of the tumor/cells and gene copy numbers, making the identification of the components of interest easier (for example, differentiation between high grade invasive ductal carcinoma with comedo-like growth pattern and comedo-like ductal carcinoma in situ) or distinguishing (Lambros et al. Hum Pathol 38:1105, 2007). In addition, in FISH analysis, tissue morphology and gene amplification are primarily disconnected because the tumor cells for signal evaluation are based on nuclear DAPI or propidium iodide staining, which does not always permit adequate histopathological evaluation of the cells (Hauser-Kronberger and Dandachi, J Molec Histol 35:647, 2004). In fact, the FISH slides have to be viewed in conjunction
with a standard hematoxylin-eosin section from the same block that will enable morphological correlations and identification of the invasive tumor (Hicks and Tubbs, *Hum Pathol* 36:250, 2005).

8 - Discussion (p13). *The sentence beginning According Wolff…’ should begin ‘According to Wolff’.*

We altered this sentence (page 16, 1st paragraph).

9 - Discussion (p14). *Mentioning that qRT-PCR has the potential to become standard is perhaps a little sweeping, in that there is little to no mention of the drawbacks: Frozen sections required, Microdissection (time consuming?), the morphology seen in CISH and IHC is lacking (a criticism levelled at FISH). These limitations should be discussed.*

We thank the reviewer for offering the opportunity to expand on these topics. We have included additional information (Background section: page 5; Discussion section: page 21, 1st paragraph).

10 - Discussion (p17), 2nd paragraph. *The sentence beginning ‘No statistically difference…’ should begin ‘No statistically significant difference’*

We altered the sentence on page 20, last paragraph, as suggested.

11 - Figure 2: *Areas 1 and 2 should be labeled.*

Figure 2 and related legend were modified as suggested.

12 - *Please add an illustrative micro photograph of the case of invasive lobular carcinoma found to have HER-2 gene amplification.*

Invasive lobular carcinomas (ILC) rarely exhibit HER-2 amplification/overexpression in most reported series (reviewed in Shackney and Shankey, *Cytometry* 29:1, 1997; Rosenthal et al. *Applied Immunohistochem & Molec Morphol* 10:40, 2002; Arpino et al. *Breast Cancer Res* 6:R149, 2004). Arpino et al. (2004) concluded that no more than 5–10% of tumors classified by a broad range of pathologists as ILC over-expressed HER-2. The patients who were positive for HER-2 may not have been of the classic ILC subtype, but possibly a variant, such as those found in the pleomorphic or mixed ILC–IDC categories. Rosenthal et al. (2002) reported that nine (13%) out of 71 cases of invasive lobular cancer showed HER-2 gene amplification by FISH analysis. The authors concluded that HER-2 gene amplification predicted disease-related death in lobular cancers overall and in LN+ lobular cancers.

In our study, one ILC showed high-level amplification by CISH, transcript overexpression by qRT-PCR and negative HER-2 immunostaining. This case of invasive lobular carcinoma was revised by the three pathologists that participated in this study (FAS, MACD, FAMN). In addition, the same cases evaluated in the present study were previously investigated for protein expression and *CDH1* promoter hypermethylation (Caldeira et al. *BMC Cancer* 6:48, 2006). The results confirmed the diagnostic of
this invasive lobular carcinoma. We added a Figure of this case showing the hematoxylin-eosin pattern, E-cadherin expression by IHC, and HER-2 amplification by CISH (Figure 2).

**Minor Essential Revision:**
Unless the paper is substantially improved and shortened.
The manuscript was edited according to the suggestions of all four reviewers.

**Quality of written English:** *Needs some language corrections before being published.*
The manuscript was revised by a British-born scientific text editor.
Figure 1. FISH analysis of HER-2 gene. A-B) Interphase disomic cells with normal pattern for HER-2 copy number showing 1-2 (A) and 3-5 (B) signals for target gene (in red) and 1-2 signals for centromere 17 (in green). (C-F) Tumor cells with HER-2 amplification demonstrating >10 signals (D) or big clusters (C, E and F) for this gene. In one case (C), cells with HER-2 amplification associated to chromosome 17 polissomy were observed.
Figure 2. Histological sections images of the invasive lobular carcinoma. (A) Hematoxylin-eosin stained section. (B) Negative E-cadherin expression by IHC. (C,D) High-level amplification of HER-2 by CISH.