Author's response to reviews

Title: SISH/CISH or qPCR as alternative techniques to FISH for determination of HER2 amplification status on breast tumors core needle biopsies: A multicenter experience based on 840 cases

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Version: 2 Date: 23 May 2013

Author's response to reviews: see over
Title: SISH/CISH or qPCR as alternative techniques to FISH for determination of HER2 amplification status on breast tumors core needle biopsies: A multicenter experience based on 840 cases

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On behalf of the participants who approved the final revised manuscript

Version: 2 Date: May 24, 2013
Author's response to reviews: see over
We thank the referees for their fruitful remarks

**Reviewer's report n°6561050059531584**

Title: SISH/CISH or qPCR as alternative techniques to FISH for determination of HER2 amplification status on breast tumors core needle biopsies: A multicenter experience based on 840 cases
Version: 1 Date: 26 March 2013
Reviewer: Haruhiko Sugimura

Reviewer's report:
The authors tested and compared qPCR, FISH, CISH, and SISH for large sets of samples. The paper is well written. The question posed by the authors well defined and practical. The content fits to the journals which has the readers involved in practice, such as lab technician and practicing pathologists.
1. The methods are appropriate and well described, but the numbers of the cases for each technology is markedly different.
2. The data are mostly sound, but some area seems to be unclear. Concordance with CISH seems to be not very high. 98% and 75% for CISH (108 and 204 cases)

*We agree that marked differences in the concordance of the CISH technique were observed between cases analysed using a dual-probe FISH assay (108 cases) and CISH cases analyzed by HER2 copy number (204 cases).*

This difference was not observed with other alternative techniques. Evaluation of the precise number of copies was difficult with the CISH technique, which must be taken into account in interpretation of the results.

3. The manuscript adhere to the relevant standards for reporting and data deposition.
4. Discussion and conclusions are well balanced and adequately supported by the data limitations of the work are clearly stated. The authors miss some previous and relevant work in minor literatures such as Kiyose S, et al. Pathol Int. 2012 Nov;62(11):728-34 and Bastien RR et al. BMC Med Genomics. 2012 Oct 4;5:44. doi: 10.1186/1755-8794-5-44.

*As suggested, the Kiyose reference has been added as n°37. We decided not to add the paper by Bastien RR (2012), as this study analyzed RNA expression by RT-qPCR, whereas we studied DNA amplification using qPCR. The purpose of our study was to discuss the various methods used to study HER2 amplification and not RNA expression.*

Level of interest: An article of limited interest

Quality of written English: Not suitable for publication unless extensively edited
Although the other referees did not indicate any problems with the written English, the manuscript has been re-edited.

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:
I declare that I have no competing interests
Reviewer's report n° 71587167953541

Title: SISH/CISH or qPCR as alternative techniques to FISH for determination of HER2 amplification status on breast tumors core needle biopsies: A multicenter experience based on 840 cases

Version: 1 Date: 26 March 2013

Reviewer: Carsten Denkert

Reviewer's report:

The manuscript by Jacquemier et al. offers data on SISH, CISH, and qPCR analysis as alternative techniques to the gold standard FISH for the determination of HER2 amplification status in a multicenter study on a large number (n=840) of paraffin-embedded core biopsies of breast tumors. The presented data shows a high concordance between all alternative techniques tested with FISH analyses. This is of special interest for the newly designed qPCR method, as the authors state that it is reliable, easy to perform and less expensive than ISH tests.

Minor Essential Revisions:

Abstract:

In the methods section of the Abstract, the number of centers in which the alternative techniques SISH and CISH were performed are given in parentheses, but the number of centers that performed qPCR is not mentioned. Another referee asked us to remove the number of centers performing each method from the abstract. We therefore suggest the following:

The number of centers performing CISH or FISH was already indicated on p5, l. 31 and 32. On p6, we have added that the qPCR method was performed in 14 centers and that the samples from the 15th center were analyzed in the coordinator’s lab without knowledge of the IHC or FISH status. One center participated in the preliminary phase of the study, but did not participate in the prospective series.

Background:

Reference 1 is missing in the text. (The first paper referred to in the introduction is the article referred to as number 2 in the reference list).

Number 1, corresponding to the 1st reference, has been added.

Methods:

1. Please give a list of antibodies used in the study. Supplementary table 1 indicates the antibodies used by the participants.
2. As the qPCR analyses are of special interest in your study, it would be advantageous to give more details on the method used, for example the sequences of the primers. It would also be interesting to learn how the data was interpreted. How did you calculate cutoff-
Were the cutoff-points pre-defined? Since the participating institutes used their own qPCR platform, did you have to define different cutoff-points for the different instruments? Supplementary table 2 indicates the primer sequences. We decided to use the cutoffs used for FISH. All participants used the same cutoffs. Most participants used Applied instruments with the same cutoff set at 0.20 for Applied 7900, for example. For the other instruments, the cutoff was adapted during the preliminary steps of the study. These details have been added to the material and methods section (qPCR).

3. Which statistical tests were used for the calculation of sample size, concordance and CI? The sample size was calculated to ensure a lower boundary of the 95% confidence interval for sensitivity with a specificity greater than 80%. This calculation was based on the formula of the 95% CI for a proportion. No statistical test was required in this study.

Results:

1. Figure 2 of the ASCO/CAP guidelines stated that patients with HER2/CEP17 FISH amplification ratio ≥ 2.0 were eligible for the trastuzumab adjuvant trials. Therefore it would be interesting to perform additional statistical analyses using a ratio ≥ 2.0 as a cutoff-point. According to the ASCO guidelines published in 2007 (ref 3 and 30), HER2 amplification status is accepted when the ratio is less than 2.2 or more than 6 copies and is considered to be borderline between 1.8 to 2.2. Additional action is required in the borderline category to reach a final decision. This means that the borderline status on core biopsies must be further investigated on a surgical specimen or, in the neoadjuvant setting, based on a multiparametric decision before considering Herceptin therapy. We know that Herceptin therapy is currently considered for a ratio cutoff ≥ 2. At the time of submission of this manuscript, we considered that the addition of another series of results with the ratio cutoff set at 2 could make the article more difficult to read and these results were therefore not included in the submitted version. However, when the results were analyzed with a ratio cutoff set at 2 and classification into 2 categories of patients, only six patients changed category for SISH and 4 for qPCR. The predictive value of the results in the overall population was very close to the initial value shown in Table 3 (ratio cutoff in 3 categories). In the 2+ subpopulation, specificity was better for qPCR using the ratio cutoff set at 2. We have added a sentence concerning these results at the end of the results section. Supplementary table 3 indicates the distribution and supplementary table 4 the indicates the predictive value of the alternative techniques compared to FISH expressed with a ratio cutoff set at 2 and we have added a sentence concerning these results in the discussion.

2. In the paragraphs on predictive value, please refer to the respective table the data is given in.

Done

3. Please include graphics on IHC and ISH staining, as well as PCR data. Three figures showing correlations between FISH and CISH, SISH and Q-PCR have been added, as requested.

Tables:

1. Recheck the formatting of Table 1 and Table 2.
2. In the legend of Table 2 it should say: “double and mono probe FISH.”

Level of interest: An article of importance in its field

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:

I declare that I have no competing interests.
Reviewer's report n° 2022631392960463

Title: SISH/CISH or qPCR as alternative techniques to FISH for determination of HER2 amplification status on breast tumors core needle biopsies: A multicenter experience based on 840 cases

Version: 1 Date: 4 April 2013

Reviewer: Merdol Ibrahim

Reviewer's report:

The authors have put forward a very nice collaborative study encompassing the protein, gene and alternative qPCR techniques on breast carcinomas showing the full range of IHC membrane expression (0,1+,2+,3+). The authors also clearly demonstrate that alternative techniques of qPCR on paraffin embedded material yield good concordance results when compared to FISH and more importantly is a technique which can be incorporated in every-day laboratory procedures.

Minor Essential Revisions

a. In the abstract; methods sections, 6th line stating “…commercially available SISH (n=10) or CISH (n=5)…”, I would suggest that the ‘n=’ numbers are removed as this appears to refer to the centre numbers rather than actual cases studied which is much higher and can lead to confusion

The number of centers has been deleted from the abstract.

b. Methods: section a) IHC and in situ hybridisation. What was the diameter of the TMA’s e.g 0.6 um? This would be of interest as TMA’s can have a tendency to show tumour heterogeneity.

A 0.6 mm diameter needle was used for TMA. This precision has been added.

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:

I declare that I have no competing interests
Reviewer's report n° 12636232489684

Title: SISH/CISH or qPCR as alternative techniques to FISH for determination of HER2 amplification status on breast tumors core needle biopsies: A multicenter experience based on 840 cases

Version: 1 Date: 14 April 2013

Reviewer: Jane Starczynski

Reviewer's report:

The determination of HER2 status has focused on the use of IHC and FISH, to look at protein and gene status respectively. It is recognised that no method is perfect for determining HER2 status or predicting response to therapy. In recent years several alternative approaches have gained acceptance including brightfield methods for evaluation gene status and molecular based approaches including MLPA and PCR. The authors have explored several of these approaches and developed and validated a PCR based method for determining the HER2 status. In parallel they have done a medico-economic study, not presented here. The study centre went through a training period to validate the methodology and the subsequent results show excellent concordance with FISH and their PCR may prove to be a viable alternative.

The study is well thought out and well presented and suitable for publication; however there are a few small areas which need further clarification or could be expanded further. These are not requirements for publication however are worthy of note. These will be addressed first and then corrections and comments that do need remedial action will be listed.

Discetionary revisions

Question 1: It is not entirely clear how the patients were selected, other than based on an expected FISH positivity. The fewest number of patients in the study were in what is probably the most difficult category, the clinically equivocal cases i.e. the HER2 2+ cases, these are the cases that require further investigation by alternate methods and can be challenging. The study may have benefitted from having more cases in this group. This is reflected in subgroup analysis where there was a broad range in interpretation amongst the study group. Are the authors planning to look more closely at patients who fall within this subgroup?

The aim of the study was to perform analyses on routine diagnostic paraffin-embedded biopsies. The number of cases was calculated by taking into account the expected FISH positivity rates, as determined by our statistician (B Esterni). No other selection criteria were used.

The results could have been more powerful with a greater number of patients in the 2+ category. As we considered that it would be difficult to work on small biopsies to obtain the required number of cases in the rare 2+ category within the timeframe of the study, we followed the statistician’s recommendations. Participating centers are currently being invited to participate in a prospective study based on 2+ cases.

Question 2: The authors have used a novel PCR technique for evaluating the HER2 status, taking both copy number and HER2/Chromosome 17 ratio into consideration. The determination of chromosome 17 number is based on the average of 5 genes on 17q,
alternative FISH approaches have used probes on both arms of 17. In view of the complexity of losses and gains that can be seen on 17 is there any reason for just selecting targets on the q arm? It maybe that this is addressed in the paper by Spyratos F, submitted for publication? This is particularly relevant for some of the more unusual patterns of loss and gain that can be seen, including co-amplification and apparent monosomy. 

We preferentially considered polysomy of the 17q arm and decided not to add one or two probes on the 17p arm. We are developing other tools with probes on the 17p arm, which could be used in the rare cases with unusual patterns mentioned by the referee.

Question 3: The CISH data adds little to this paper. There are less than half of the cases than in the other methods presented, this could skew the statistics. The authors go on to say that there is a move away from this technique.

CISH was the alternative technique chosen at the beginning of the study, but due to variations in the duration of inclusion of cases from one center to another, few centers used CISH as alternative technique, as SISH was rapidly found to be faster and easier to use.

Required Actions

P4: Please reference the statement “Therapeutic response to trastuzumab was observed exclusively in patients harbouring HER2 gene amplification”
The Perez reference has been added in position 12.

P4: “...and is also expensive”. Is FISH any more expensive on a cost per test basis that the brightfield alternatives? This statement is equally applicable to both.

Due to the investment in terms of processing time and the expensive fluorescent microscope needed to read FISH, we considered that this technique constituted a major limitation to evaluation of HER2 amplification status. Work is in progress concerning the medico-economic assessment of the various alternative techniques.

P5: The authors talk about SISH, this methodology has evolved into several versions please clarify that all centres were using the two slide method with separate HER2 and CEP 17 and not one of the dual colour versions such as DDISH.

At the time of this study, only the two-slide method was used for SISH.

P5: Please review the statement on ASCO/CAP guidelines. The authors state 1.8-2.2 borderline amplification, this is not what ASCO/CAP calls this equivocal, however with patients >2.0 eligible for trials.

According to the ASCO guidelines published in 2007 (ref 3 and 30), HER2 amplification status is accepted with a ratio less than 2.2 or more than 6 copies and is considered to be borderline between 1.8 to 2.2. Additional action is required in the borderline category to reach a final decision. This means that the borderline status on core biopsies must be further investigated on surgical specimen or, in the neoadjuvant setting, based on a multiparametric decision before considering Herceptin therapy.

We know that Herceptin therapy is currently considered for a ratio cutoff ≥ 2. At the time of submission of this manuscript, we considered that the addition of another series of results with the ratio cutoff set at 2 could make the article more difficult to read and these results were therefore not included in the submitted version. However, when the results were analyzed with a ratio cutoff set at 2 and classification into 2 categories of patients, only six patients changed category for SISH and 4 for qPCR. The predictive
value of the results in the overall population was very close to the initial value shown in Table 3 (ratio cutoff in 3 categories). In the 2+ subpopulation, specificity was better for qPCR using the ratio cutoff set at 2. We have added a sentence concerning these results at the end of the results section. Supplementary table 3 indicates the distribution and supplementary table 4 the predictive value of the alternative techniques compared to FISH expressed with a ratio cutoff set at 2 and we have added a sentence concerning these results in the discussion.

P6: Standardize more appropriate word than homogenize. Done

P9: variable expertise rather than heterogenous, a little misleading when talking about HER2. Done

Level of interest: An article of importance in its field

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:

I declare I have no competing interests.