Author's response to reviews

Title: Pregnane X receptor is expressed in human breast carcinomas. Potential heterodimer formation between PXR and RXR-alpha

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Author’s response to reviews: see over
Dear Sir,

Herewith I am enclosing the revised version of the manuscript entitled PREGNANE X RECEPTOR IS EXPRESSED IN HUMAN BREAST CARCINOMAS. POTENTIAL HETERODIMERS FORMATION BETWEEN PXR AND RXR-α (I Conde, MVT Lobo, J Zamora, J Pérez, FJ González, E Alba, B Fraile, R Paniagua, MI Arenas), which has been made taking into account the referees’ reports.

We are enclosing text, figures, together with a letter of explanation of changes made following the referees’ concerns.

I hope that the revised manuscript merits now publication in BMC CANCER.
LETTER EXPLAINING THE CHANGES MADE FOLLOWING THE REVIEWERS’ COMMENTS

Reviewer 1
1. They showed PXR to be associated with a postoperative recurrence in 38 patients. PXR might be a postoperative prognostic factor, but not a predictive factor.
This item has been modified in both, the Conclusion of the Abstract and in the Discussion of the manuscript (Page 12, lines 8-9).
2. The terms of first group and second group should be defined. How many patients underwent postoperative endocrine therapy or chemotherapy? Did all patients undergo only endocrine therapy?
This paragraph has been rewritten (page 4, lines 17-28) the two patients’ groups are defined taking into account the appearance of recurrence. In the first group (without relapse), 19 patients received adjuvant therapy with tamoxifen and 20 patients were treated with tamoxifen and chemotherapy, 5 with chemotherapy only and 1 of them received radiotherapy. In the group 2, 3 patients received tamoxifen therapy and 19 chemotherapy and tamoxifen, 4 chemotherapy only and 5 adjuvant endocrine therapy without tamoxifen.
3. Analysis for postoperative recurrence should be using survival curve analyses such as Kaplan Meier method with a log-rank test.
This analysis has been done; see the Results section (page 8, lines 21-22, 26-28 and Figs. 4 and 5).

Reviewer 2
Major Compulsory Revisions. The authors must significantly increase the size of the cases analyzed in order to give more strength to their data
During the last month, we have got thirty nine new samples of infiltrative carcinomas and were included in this study, 25 of them were ductal infiltrative carcinomas and fourteen lobular infiltrative carcinomas. In this period, we have done immunohistochemistry with these samples, we have reviewed clinical records and samples were included in the new statistical analysis showed in the Results.
Manuscript needs some language corrections before being published.
The manuscript has been revised by an English-speaking colleague; however, we would thank for the editorial assistance.
Reviewer 3

1. The authors should compare the result of this manuscript and previous reports such as Miki’s report and Dotzlaw’s report, respectively.

The reports by Dotzlaw et al. and Miki et al. have the numbers 14 and 18 respectively in the References. Our results were compared with their results in the Discussion section (page 9, lines 4-8; page 10, lines 1-4).

2. What about the function of nuclear receptor SXR/RXR in cytoplasm?

The nuclear and cytoplasmic location of PXR is discussed on page 9, lines 23-26 and also in page 10, lines 5-15. The biological significance of the differential location is yet unknown.

The authors also should consider the significance of the correlation between cytoplasm PXR and nuclei RXR.

The statistical significant correlation between cytoplasmic PXR and nuclear RXRα indicates that, in infiltrative carcinomas, the expressions of these receptors are related. Since RXRs are needed partner of other receptors such as RAR, VDR, PPAR, CAR, etc., the nuclear presence of RXRα may also indicate the heterodimerization with a nuclear receptor different to PXR.

3. In this manuscript, the authors confuse human PXR, which named SXR with rodents PXR. The author should distinguish SXR reports and PXR reports in the sections of Introduction and Discussion.

This item has been emended in this revised version of our manuscript.

Methods

Patients and histological samples:

4. Informed consent should be obtained from all patients. Did the ethics committee at your university approve this research protocol?

As it is described in the Material and Methods section (page 4, lines 4-5), the samples were used with the consent of the patients and permission of the Ethics Committees of the two University Hospitals. In addition, we obtained the approval of the Ethics Committee at our University.

5. Invasive ductal carcinoma includes special-type carcinoma or not?

In the selection of samples we didn’t include the special-type of carcinomas in order to eliminate changes due to the ethiology of the type of carcinoma.

Immunohistochemistry

6. How to evaluate PXR immunoreactivity is unclear. In generally, labelling index is
used as the evaluation for immunohistochemistry of nuclear protein. The immunoreactivity for PXR and RXR should be scored as labelling index.

In reports considering the immunoreactivity of nuclear receptors in human breast cancer tissues, authors use the Allred system established for ER receptors although with a variation in the cutoff used to define positivity (Carder et al, 2005). Normally, the cutoff point was defined as nuclear staining in more than 10% of the cancer cells. Despite of these criteria the lack of a standardized scoring method, especially for ER-β, is recognized. And, this happens in the most of the manuscripts that have focused their studies on a long time known ERβ receptor. These discrepancies in reactivity are due to the antibodies, the antigen retrieval method or the buffer used. However, in these papers the cytoplasmic reactivity was not considered and although many authors assume that this location reflect genuine expression rather than an artefact, they did not value it. Considering the former reasons, we adopted as nuclear staining the positivity in more than 10% of the cancer cells without categories, because we have not observed any variation in the immunostaining intensity; and, in contrast, we established two categories to evaluate the cytoplasmic intensity.

7. For negative control, immunohistochemical preabsorption test are also necessary for verifying specify of polyclonal antibodies used in this study.

We used as negative controls, sections of breast samples processed identically and incubated using the antibody preabsorbed with corresponding blocking peptide (page 5, lines 4-5). This was already described in the text of our original manuscript.

Results and Figures

8. Western blotting. There were multiple bands in PXR analysis. The authors should suggest that these bands are correct or extra bands.

Since Dotzlaw et al. reported the presence of hPXR mRNA in human cancer cells lines, we have used extracts of MCF-7 and MDA-MB-237 cells to control the antibodies used and we have compared the pattern bands observed in these cell lines with that obtained in human tissues. The presence of these multiple bands is discussed on page 9, lines 9-19.

Results and Figures

9. Immunohistochemistry. Positive control and negative control should demonstrate in figure.

In this revised version a new figure showing negative and different positive controls has been added (See Figure 2).
Discussion

More extensive discussions described above and the following points are needed.

10. CYP3A4 is considered the main SXR-regulated gen. But, the authors did not examine its expression pattern in breast disorders in this study. Recently, other metabolic enzyme and transporter were also reported to be regulated by SXR.

The discussion section has been extended. We discuss the interaction between CYP450 and PXR on page 11, lines 21-30. In this work, we have focused in the expression of PXR and RXRs in human breast tissues. Thus, the analysis of expression of CYP3A4 in breast tissue is out of the objectives of this study.

11. The expression of RXRs in breast cancer cell lines.

We have discussed the expression of RXR receptors (see page 10, lines 16-33 and page 11, lines 1-8).

12. The functions of PXR2 and cytoplasm PXR/RXRs.

The nuclear and cytoplasmic location of PXR is discussed on page 9, line 5-15 and on page 10, lines 24-29. The biological significance of this different location is yet unknown. Taking into account the results showed in this manuscript, we started to analyze the mechanisms of the PXR activation by different compounds in breast cancer cell lines.

Reviewer 4

1. In Table 2, authors described that the significant difference between PXR and cytoplasmic expression of RXR beta and gamma as well as between PXR and nuclear expression of RXR alpha. The authors should discuss it.

This item has been discussed; see discussion section, page 10, lines 16-33 and page 11, lines 1-8.

2. Authors described that the PXR expression was inversely correlated with the prognosis. Because PXR is thought to play some roles in the metabolism or endogenous steroid hormones and drugs, authors should mention the potential roles in the prognosis more.

Taking into account this suggestion, the discussion has been extended (page 11, lines 21-30).

3. In conclusion, author mentioned that PXR-RXR-alpha heterodimers might be involved in the development of endocrine therapies resistance. If so, the PXR and RXR-alpha expression in relapsed tissues after endocrine therapy should be examined.
Samples used in this study were primary tumours from patients who did not receive neither radiotherapy nor chemotherapy or neoadjuvant therapy before surgery. The recurrence observed in some of patients was metastasis in liver, bone or brain and in these cases they received a second line of treatment without surgery; therefore we couldn’t get new relapse samples. In addition, we have focused this study in the expression of these receptors before any treatment to check if they might be useful for prognosis.

4. The relationship between RXR expression and clinicopathological data in these cases should be examined to clarify the importance of RXR alpha in breast cancer prognosis.

This has been done; the results are expressed in Tables 4 and 5 and described on page 8, lines 23-28.

5. Some reports have described the localization of PXR might be affected in the presence or absence of ligands. The mechanism of different PXR localization among benign, carcinoma in situ and advanced tissues should be discussed. Also the additional experiments to clarify this mechanism should be studied using breast cancer cell lines.

The nuclear and cytoplasmic location of PXR is discussed on page 9, lines 33-34 and page 10, lines 1-15. The biological significance of this different location is yet unknown; however, the present manuscript shows a retrospective study in human breast cancer, we reviewed the patients’ medical history after the immunohistochemistry analysis since our objective was to analyze the differences of PXR expression in breast tissues. Taking into account the results showed in this manuscript, we started to analyze the mechanisms of the PXR activation by different compounds in breast cancer cell lines; however, these experiments belong to a different set of objectives separated from this manuscript.

6. Manuscript needs some language corrections before being published

The manuscript has been revised by an English-speaking colleague; however, we would thank for the editorial assistance.