Author's response to reviews

Title: Sialyl Lewis x expression in canine malignant mammary tumours: correlation with clinicopathological features and E-Cadherin expression

Authors:

Salome S Pinho (salomep@ipatimup.pt)
Augusto J F Matos (ajmatos@icbas.up.pt)
Célia Lopes (celiacristinalopes@gmail.com)
Nuno T Marcos (nmarcos@ipatimup.pt)
Julio Carvalheira (JGC3@mail.icav.up.pt)
Celso A Reis (celso.reis@ipatimup.pt)
Fatima Gartner (fgartner@ipatimup.pt)

Version: 2  Date: 28 March 2007

Author's response to reviews: see over
To: Doctor Deborah Saltman
   Editorial Director of BMC Cancer

Dear Sir:

We are submitting the revised manuscript entitled “Sialyl Lewis x expression in canine malignant mammary tumours: correlation with clinicopathological features and E-Cadherin expression”, in light of the reviewers’ comments. We provide a cover letter giving a point-by-point response to the reviewers’ concerns.

Reviewer: Hiroyuki Taniyama

Which concerns to the introduction we agree with the reviewer and we shorten the introduction. We moved to discussion most of the first paragraph in page 5.

Accordingly with the reviewer, in Histological examination of the tumours (page 7, Material and Methods) we cut off some information that was already described in a published paper of our group [Reference 33]. We think that the first paragraph (Page 7, Line 2 to Line 7) constitute a relevant information to be provided in this section.

The follow-up data described in page 7 constitute important information about all the animals included in this study. This information represents the material that was used in this study to obtain the distant metastases data that was related with sLe\(^x\) expression. So, we think that it is more appropriate to provide this information in Material and Methods instead of Results.

In page 7 (Immunohistochemistry section, first paragraph) we delete some repeated information, accordingly with the reviewer.
Reviewer: Juana Martin de las Mulas

Major Compulsory Revisions

1. As it was described during the manuscript, sLe\textsuperscript{x} is a carbohydrate antigen associated with malignant phenotype. An altered cell surface glycosylation that occurs during malignant transformation leads to the expression of sLe\textsuperscript{x}. Because sLe\textsuperscript{x} was been associated in many types of human cancers only with malignancy, this is the reason why we focus our attention only in carcinomas. Although, in all cases we have also took into account the adjacent benign proliferative lesions, which showed to be negative for sLe\textsuperscript{x} expression. Only the mammary gland secretion showed some positive staining for sLe\textsuperscript{x}. Accordingly to the reviewer suggestions we added a sentence regarding the evaluation of benign proliferative lesions (Page 13, Line 2 to Line 6).

2. The expression of sLe\textsuperscript{x} in \textit{in situ} carcinomas and in carcinomas in benign tumour are represented in table 1 (page 28). Taking into account the comments of the reviewer we included a sentence that describes the sLe\textsuperscript{x} expression in those tumours and a sentence that refer in which cellular component of canine complex and mixed mammary carcinomas (epithelial cells) was observed expression of sLe\textsuperscript{x} (Page 12, “Expression of Sialyl Lewis x in canine mammary carcinomas” section).

3 and 4. Accordingly to the reviewer suggestion we added more clinical information about the follow-up data (page 12 Line 13 to Line 16). However, all follow-up data from the animals that were included in this study are still been analysed by our group to be a part of a new manuscript with the major aim of studying prognostic factors (paper already submitted for publication). Besides, to study the importance of sLe\textsuperscript{x} in canine mammary carcinomas we chose distant metastases from all follow-up data because sLe\textsuperscript{x} has been closely associated with hematogenous metastases in many types of cancers.

5. We state that sLe\textsuperscript{x} could be used as a prognostic tumour marker in canine mammary carcinomas based on results that not only demonstrate positive
expression of sLe\(^x\) (that constitute a carbohydrate closely associated with malignant transformation) either in sites of squamous metaplasia (used as a criteria of malignancy in canine mammary gland tumours) but also we demonstrate a significant association of sLe\(^x\) expression with the presence of lymph node metastases. The presence of lymph node metastases in an animal with mammary carcinoma leads to a third clinical stadium classification of the animal, which in turns means that the tumour has metastatic potential and consequently poor prognosis. So, as other described tumour prognostic markers, the significant relationship between sLe\(^x\) expression and lymph node metastases, in our point of view seems to be sufficient to classify sLe\(^x\) as a valid prognostic factor.

6. As we reported in page 9 (Line 10 to Line 15), the expression of sLe\(^x\) was classified in the following manner: negative; < 25%; 25-50%; 50-75%; > 75%. Because the majority of the tumour cases (60,4%) revealed < 25% of sLe\(^x\) expression, our statistician define the cut off in 25%. So, for statistical analysis the expression of sLe\(^x\) was regrouped in two percentual categories (< 25% and ≥ 25%) in order to increase the number of cases in each category and improve the statistical power of the tests, as we described in page 9 (line 16 to Line 21).

7. Our results showed an inverse relationship between sLe\(^x\) and E-cadherin expression (stated in page 14, Line 4 to Line 13). When we analysed by double labelled immunofluorescence the simultaneous expression of both adhesion molecules in the same tumour section we could observe that cells that express E-cadherin are negative for sLe\(^x\) and vice-versa (stated in page 14, Line 14 to Line 18). The reason behind this “incompatibility” of simultaneous expression of sLe\(^x\) and E-cadherin is not yet clearly understood in the veterinary field, not even in human cancer and constitute the aim of our further investigations. In page 18 we discuss the possibility of these two molecules to play opposite roles in the same tumour cell. We hypothesise that it could be an internal mechanism of the tumour cell that regulates the expression of sLe\(^x\) and E-cadherin. In addition it is not clear that alterations in one cell may induce a change in another cell, but rather we favour a mechanism in the same tumour cell.
Accordingly, we added a sentence that addresses our hypothesis in page 18, Line 23 to Line 25 according to the reviewer suggestion.

8. Taking into account the reviewer comment we put picture A and B (Figure1) with the same magnification. In Figure 2 we added arrows that could help interpretation.

**Minor Essential Revisions**

Page 6: We agree with the reviewer. We used the term “large series” just because up to now the study of sLe\(^\times\) in canine mammary carcinomas was made in a small series of malignant tumour. We delete the term “large” (page 6, Line 5 to Line 10).

Page 12, Line 1: We corrected the sentence.

---

**Reviewer: Cinzia Benazzi**

**Major Compulsory Revisions**

Page 5, Line 19: as requested by the review we have added a reference.

Page 5, Line 22: As the best of our knowledge only the Barsky’s group describes the cooperation between E-cadherin and sLe\(^\times\) using only a mouse model of Inflammatory Breast Cancer (MARY-X) [Reference 30-32]. More recently, Jeschke et al identified a negative correlation between Sialyl Lewis antigens and E-cadherin in woman breast cancer (they compare the expression of both molecules using *in situ* carcinoma, invasive carcinomas (ductal and lobular) without lymph node metastases and invasive carcinomas (ductal, lobular and mucinous) with lymph node metastases [Reference 23].
Page 6, Line 6: We agree with the reviewer. We used the term “large series” just because up to now the study of sLe^x in canine mammary carcinomas was made in a small series of malignant tumour. We delete the term “large”.

Page 6, Line 9: In fact the results of the immunohistochemical study of E-cadherin expression in these series of tumour cases were already published by our group. Taking into account the reviewer comment we put the citation of that study when we mention expression of E-cadherin [Reference 33].

Page 6, Line 13: 102 local and regional lymph nodes were excised. As requested by the reviewer we added the number of local and regional lymph nodes that were excised.

Page 6, Line 20: The immunohistochemical study of lymph nodes was already discussed in our published paper [Reference 33]. It was performed using the following primary antibodies: anti-pancitokeratin antibody AE1/AE3 and anticytokeratin 14. We have used these previously published information results in this manuscript and have added the proper reference in the text.

Page 8
The study of the immunohistochemical expression of E-cadherin in these series of cases was already published by our group. This is the reason why we did not described in detail the method used for immunohistochemical study of E-cadherin. A reference has been added.
We used the conventional scoring method described by other authors in other studies to analyse the immunohistochemical staining of tissue sections.

Page 11
We agree with the reviewer. Our results were based on evaluation of the immunoreactivity under only one microscope (Leica DMIRE2) and we have corrected it on the manuscript.

Page 12 (line 4 to Line 10): We added the followed sentence: “The majority of the cases that showed lymph node metastases were classified as
carcinosarcomas (6 out of 11). The others were classified as complex carcinoma, tubulopapillary carcinoma and solid carcinoma. From the eleven cases that revealed nodal metastases, 4 patients revealed in addition, distant metastases. Seven patients with lymph node metastases died (in 4 animals was performed euthanasia because the tumour and 3 animals died of other causes).” Taking into account the classification of tumours that showed lymph node metastases, the morphological features observed in lymph node metastases were similar to the primary tumours.

Page 12, Line 23: The reviewer comment was in agreement to what we expected to observe: sLe\(^x\) positivity in the cell membrane. However, we could not rule out that in cancer cells some cytoplasmic accumulation of sLe\(^x\) may occur leading to observation of some cytoplasmic and/or membranous expression of sLe\(^x\).

Page 13, Line 2: To be more clearly we rephrased the sentence.

Page 16, Line 9: Some authors use squamous metaplasia as criteria for malignancy diagnosis in canine mammary gland tumours [Reference 44]. Tumours that exhibit squamous metaplasia have more probability to be malignant than tumours that do not exhibit this feature. Squamous metaplasia does not tell us anything about the grade of malignancy. sLex was been associated with malignant phenotype of different types of tumours including these of canine mammary carcinomas. What we observed was that sLex was always expressed in sites of squamous metaplasia which reinforce the use of sLex in the malignancy diagnosis of canine mammary gland tumours.

Page 18, Line 13: We agree and therefore included the references suggested by the reviewer.

To the best of our knowledge, the relation between E-cadherin and sLe\(^x\) expression was new in canine cancers particularly in canine mammary carcinomas. This is the first report of such a relationship. The reason behind this “incompatibility” of simultaneous expression of sLe\(^x\) and E-cadherin is not
yet clearly understood in veterinary field and in human cancer. In page 18 we discuss the possibility of these two molecules to play opposite roles in the same tumour cell. We think that it could be an internal mechanism of the tumour cell that regulates the expression of sLe$^x$ and E-cadherin. The clarification of this inverse correlation from the biochemical point of view is imperative and, as we mention in conclusions, constitutes the aim of our investigations. Accordingly with the reviewer we added a sentence that addresses our hypothesis in page 18, Line 23 to Line 25.

Page 20
We included DAPI in list of abbreviations

Table 1
We added the P values as requested by the reviewer.
The second question was answered above.

Minor Essential Revisions

Page 6, Line 17: We rephrased the sentence

Page 7, Line 9: We correct it
We added the information about the number of dogs dead or alive at the end of the follow-up period in page 12.

Page 10, Line 3: We correct the verb.

Page 11
We correct it

Page 12
Line 11: We rephrased the sentence

Page 12
Line 18: We indicate the method used: immunohistochemistry.
We correct the tenses

One of the reviewers suggests shorting the introduction and moved some information to the discussion. Accordingly with both reviewers’ comments we moved the description of sLe\textsuperscript{x} from the introduction to the discussion.

We added references

We think that the sentence stated in Line 9 to Line 14 helps the interpretation of the results.
We moved the sentence to conclusions

We reorganize the conclusions

Point 18: We correct it.

Taking into account the reviewer comment we put picture A and B (Figure 1) with the same magnification.

In Figure 2 we added arrows that could help interpretation. The expression of sLe\textsuperscript{x} in squamous metaplasia was observed either in the cytoplasm and cell membrane. The figure 2 only reveals sLe\textsuperscript{x} in the cytoplasm. The explanation for that fact was the same for other sites of the tumour: it could be possible to occur cytoplasmic accumulation of sLe\textsuperscript{x} accompanied with the malignant transformation.
Figure 3
To evaluate the relationship between sLe\(^x\) and E-cadherin expression in the 53 tumour cases we performed immunohistochemistry followed by statistical analysis of those results. With the purpose of documentation and to confirm such a relationship we performed double labelled immunofluorescence.

Figure 4
Both methods (immunohistochemistry and immunofluorescence) stain epithelial cells in cell membrane and/or in the cytoplasm. The figure 4 only shows expression of sLe\(^x\) in the cell membrane.

Sincerely yours