Title: Mutually exclusive expression of DLX2 and DLX5/6 is associated with the metastatic potential of the human breast cancer cell line MDA-MB-231

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Author's response to reviews: see over
Dear Editor,

Enclosed please find the revised copy of the manuscript # 8093672529420711: “Mutually exclusive expression of DLX2 and DLX5/6 is associated with the metastatic potential of the human breast cancer cell line MDA-MB-231” by Morini M., Astigiano S., Gitton Y., Emionite L., Mirisola V., Levi G. and myself.

Following the indications of the Referee #2 we have modified the Title.

**Referee #1 did not have any further objections.**

You can find below the answer to Referee #2 and Referee #3.

**Referee # 2.**

**Major compulsory revisions:**

1. The Authors propose that the switch between DLX2 and DLX5/6 expression is associated to the metastatic potential of breast cancer cells and that DLX5 expression is a negative prognostic marker in this disease. Considering DLX5+ patients represent 2.2% of the whole casistic, this latter conclusion should be supported by a rigorous statistical analysis to take into account the characteristics of the patients and known prognostic variables in breast cancer.

   **Answer:** In the original text of the paper we had already stated (Page 13, 6 lines from the bottom) that the comparison between the small group of Dlx5+ patients and the other groups was non-significant due to the small size of this sample. The p-values had already been introduced in Fig. 5D. To make this statement clearer and to take into account also the observation of Referee #3, we have also considered the age of patients as a co-variant. We have reformulated the sentence which now reads: “Patients were then divided into three groups, i.e. DLX2+/DLX5-, DLX2-/DLX5+ and DLX2-/DLX5-, and disease-free survival and relapse were assessed. The DLX2+/DLX5- patients had the longest average disease-free survival, whilst the DLX2-/DLX5+ patients had the shortest one. Kaplan-Meier analysis of disease-free survival showed a significant difference between the DLX2+/DLX5- and DLX2-/DLX5- groups (p=0.005), however, the very small size of the DLX2-/DLX5+ sample resulted in non-significant difference when comparing this group of patients with either the DLX2+/DLX5- or the DLX5/2- groups (Fig. 5C and D). Taking into account the age of patients as a co-variant did not modify these results.” The abstract has also been changed to make this point clearer.

2. The validation set is still missing. I feel that the validation of the results even in a relatively small but representative number of primary tumors and nodal metastasis is of fundamental importance to determine the existence of the switch in humans and, most importantly, the clinical relevance of DLX5 expression.

   **Answer:** The experiments were designed on animal model, and we used the human breast microarray data set in the attempt to understand if human breast cancers behave alike. Our data show that Dlx5 is highly expressed only in a small subset of tumors with a particularly bad prognosis, which, due to the small size of
this sample, did not show a significant difference from the other patients (as already stated in the original version of the paper). We feel that “few significant cases” would not change the significance of our findings. In order to demonstrate the involvement of Dlx5 in metastasis formation in human breast cancer we would need a very high number of samples. As a matter of fact, in the paper we suggest to consider Dlx2 and Dlx5 as potential prognostic markers, provided that our results are confirmed in a different cohort of patients. Moreover, following the suggestion of the first referee, we made a bad choice of the title that was indeed misleading. Thus, as requested, we changed it again to “Mutually exclusive expression of DLX2 and DLX5/6 is associated with the metastatic potential of the human breast cancer cell line MDA-MB-231”, that better represents the essence of our work.

Additional points:
- Although the new title: “Mutually exclusive expression of DLX2 and DLX5/6 is associated with the metastatic potential of breast adenocarcinoma” was proposed by Reviewer 1, we do not fully agree with this choice. Indeed the association between DLX switch and the metastatic potential is demonstrated only in the mouse model with a single cell line and no data on human metastasis (i.e.: involved nodes) are present.
  Answer: Done. See above

- DLX6 expression is limited to the in vitro and in vivo experiments in the mouse model. Importantly, the in silico analysis show that DLX6 expression does not differ between normal vs tumors, so its relevance should at least be discussed.
  Answer: Regarding Dlx6 expression, we had already addressed the topic in the Discussion section, at page 15, line 3.

- In the survival curves of Figure 5 the patients are subdivided into three groups to consider all the possible variants of DLX2 and 5 expression (DLX2+/5-, DLX2-/5- and DLX2-/5+). In fig.6 the patients are divided in DLX5 “high” and “low” (presumably DLX5 + and -) and put in relation with ET1 expression. Since ET1 is an inhibitor of DLX2 and an inducer of DLX5/6, the patients in Fig. 6 should be subdivided as in Figure 5 and the results should be commented.
  Answer: After applying the statistical analysis suggested by Referee#3 we have now removed Figure 6 (see later). Indeed, in our model ET1 induction should take place in the tissue targets of metastasis and not in the primary tumor therefore the analysis of ET1 expression in this dataset is not informative and it is actually confusing.

- In the response letter, the Authors state that in situ hybridization on bone metastases was not performed with DLX2 probes because these metastases resulted negative by PCR. The data should be reported in the text to define the general phenomenon of DLX switching in the mouse model.
  Answer: The relative statement was already present at page 11, line 13.

Referee #3

General remarks
As remarked by the reviewer Barbara Banelli, a proper survival analysis using e.g. a
Cox-regression controlling for at least age and possibly also other confounding factors is needed.

**Answer:** As mentioned before we already stated that the difference between the Dlx5+ group and the other was not significant, we have now performed an analysis controlling for age effects and we did not find any change in our results.

**Specific remarks**
- The estimates should be given as rate-ratios — that is what comes out of a Cox-regression. Confidence intervals should be given for the estimate rate-ratios, and they should be controlled for possible confounders.

**Answer:** In the Figure 5 we added the 95 % Confidence Intervals. As mentioned above analysis for age effects did not change the results.

- The relative risks are labeled wrong — the bottom one is DLX5+ vs. DLX2/5.

**Answer:** The mistake has been corrected.

- The analysis of the ET1 expression in the DLX5+ groups is too rudimentary. Fromm the boxplot one would suspect that a log-transform would be required to make a proper t-test of whether the two means were equal. The p-value from the t-test could probably not really be trusted, so a randomization p-value should be computed: 1) Compute the t-test based on the existing data for ET1 and DLX5. 2) Randomly assign 9 patients to one group and 399 to another, and compute the t-test. Repeat say 10,000 times. 3) The fraction of instances where the simulated date gives a higher t-statistic than the existing data is the randomization p-value.

**Answer:** We thank the reviewer for his useful comments. We followed exactly his suggestions: to compute a randomization p-value we randomly assigned 9 patients to one group and 399 to another, and compute the t-test. We repeated this procedure 10,000 times and we evaluated the fraction of instances where the simulated date gives a higher t-statistic than the existing data. We obtained randomization p-value = 0.21. Therefore we conclude that there is no significant correlation between ET1 and DLX5 expression. As mentioned before, this fits our model which suggests that ET1 induction should take place in the tissue targets of metastasis and NOT in the primary tumor. We have now removed Figure 6 and the associated text.

We thank you for your kind attention, and look forward to hearing from you soon.
Sincerely Yours,
Ottavia Barbieri