Author's response to reviews

Title: Intratumoral heterogeneity impacts the response to anti-neu antibody therapy

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Author's response to reviews: see over
Ms: 7127021251158884

Title: Intratumoral heterogeneity impacts the response to anti-neu antibody therapy.

Thank you for your kind reviews. I tried to follow all of reviewer's recommends. And follows are our responses and changed points of manuscript.

I. Responses to reviewer’s recommends.

Reviewer: Lucas Abrahao-Machado

1. The assessment of HER2 status either by immunohistochemistry or by FISH is essential in this study. To demonstrate the intratumoral heterogeneity, which is in my opinion the most important subject of this paper, the authors could use immunohistochemistry, the simplest method and very accurate. I think it is not very clear how intratumoral heterogeneity of HER2 was assessed.

: As reviewer’s recommend, we assessed tumor heterogeneity using immunohistochemistry and inserted results at Figure 4A. By increasing ratio of TUBO-P2J, Neu-negative cells were increased. IHC for neu antigen showed heterogeneity of HER2/neu. Description about IHC results were inserted from line 7 to 16 of p17.

2. The introduction is confused. The term antibody is equivocal since it can be used to refer both as immunohistochemical antibody and as the drug. i suggest replacing the word antibody for drug.

: Sorry for making confusion.

In introduction, the word antibody was appeared at 3 places; line 5 of p6, line 2 and line 9 of p7.

Antibody at line 5 in p6 is hard to replace drug, because is explain the trastuzumab.
Antibody at line 2 and 9 in p7 were removed.

Reviewer: paulcottu

1. The authors should be cautious: they have a general trend along the manuscript to
overinterpret the results. For example, they present no validation data supporting the assertion that TUBO-P2J invasion capacity may be attributed to MMP9 expression (e.g. knock out or SiRNA data). There is also no formal demonstration that the expression of mesenchymal markers is these cells is associated with an effective EMT process.

: Thank you for kind recommends. We tried to remove overinterpretation and add data about that.

1) no validation data supporting the assertion that TUBO-P2J invasion capacity may be attributed to MMP9 expression.
► We evaluated migration and invasion with MMP9 inhibitor I. The results showed that MMP9 inhibitor blocked invasion but did not affect migration. This data inserted in Fig 2H and Supplementary Fig 2. Descriptions were inserted in line15-22 of P15.

2) the expression of mesenchymal markers is these cells is associated with an effective EMT process.
► This criticize might be caused by wrong sentence at line 22-23 of p16.
   We just want to clear that TUBO-P2J cell showed the phenotype of mesenchymal cells and TUBO cells did that of epithelial cells. So, the sentence was changed.
: These data suggest that the EMT process potentiates the metastatic capability of TUBO-P2J. These data suggest that the EMT process might be related in phenotype changes of TUBO cells to TUBO-P2J cells.

2. The heterogeneity chapter is difficult to understand as well. The authors present an experimental “heterogeneous” HER2+ tumor, based on a mix of TUBO and TUBO-P2J cells at 2 different dilutions. However and this is a critical limitation to their results, they present no data proving that HER2 expression is indeed heterogeneous in the resulting xenograft. A relationship between tumor cell dilution and extent of HER2 heterogeneity in the final tumor should also be established. These data are required before reconsidering the manuscript. Additional cell dilutions would be also welcomed.
: We proving heterogeneity of mixed tumor with IHC for HER2/neu antigen. These data showed that mixed tumors were composed with Neu-positive and –negative cells and ratios of Neu-negative cells were increased by increasing ratio of TUBO-P2J cells when
tumor implantation.

Data was inserted in Fig 4A.

3. The origin of the TUBO cell line should be indicated.
   : Inserted description about the origin of TUBO cell line at Methods, line 10 of p8.

4. IHC data are required for comparative HER2 status assessment in TUBO and TUBO-P2J cell lines.
   : Data was inserted in Fig 4A.

II. List of changes during revision.
1. P 6, line 2: deleted anti-neu antibody therapy anti-neu therapy
2. P6, line 9: antibody based therapy anti-neu therapy
   : TUBO was cloned from a spontaneous mammary tumor in a BALB Neu Tg mouse [10].
4. P7, line 15-16: inserted sentence about MMP9 specific inhibitor
   : MMP9 specific inhibitor (CAS 1177749-58-4, IC_{50} for MMP9 = 5 nM, IC_{50} for MMP1 = 1.05 µM) was purchased from SantaCruz.
5. P11, line2-9: inserted methods for IHC.
   : Tumor tissues were fixed in 4% paraformaldehyde and then were embedded in paraffin blocks. Tissue sections from a paraffin block (4 µm thick) were incubated in tris-EDTA buffer (pH 8.0) and heated to 99°C for 30 min. After the endogenous peroxidase activity was quenched with 3% hydrogen peroxide, the sections were treated with UV inhibitor (Ventana, CA, USA). The sections were incubated with biotinylated anti-neu antibody (7.16.4) at 37°C for 30 min. The sections were incubated with then streptavidin-HRP (BD Biosciences) for 8 min. Finally, counterstaining was performed with Mayer’s hematoxylin.
6. P15, line 15-20: inserted sentences about MMP9 inhibitor experiments
   : To test whether the active MMP9 expression caused increase of migration and invasion, MMP9 inhibitor I (CAS 1177749-58-4) was used. Invasion of TUBO-P2J cells was reduced to more than 95% by 5 nM of MMP9 inhibitor I (IC_{50} for MMP9) and blocked completely by 50 nM (Fig. 2H). However, migration of TUBO-P2J cells was not changed by MMP9 inhibitor (Supplementary Fig 2).
These data suggest that the EMT process potentiates the metastatic capability of TUBO-P2J. These data suggest that the EMT process might be related in phenotype changes of TUBO cells to TUBO-P2J cells.

We then evaluated whether these mixed tumors showed intratumoral heterogeneity and how these heterogeneous tumors responded to anti-neu antibody treatment in a neo-adjuvant setting (Fig 4). After transplanted tumors were established (50-100mm³), intratumoral HER2/neu heterogeneity was evaluated with IHC using biotinylated anti-neu antibody (7.16.4) and streptavidin-HRP. However positive cells were not mainly stained on membrane, positive and negative cells were easily discriminated. Mixed tumors were composed with Neu-positive and –negative cells and negative cells were increased by increasing ratios of TUBO-P2J (Fig 4A). Although the percentages of Neu-negative cells were higher than initial mixed ratio, Neu-positive cells were more than 50% in 0.1% mixed tumors.