Author's response to reviews

Title: Impact of complex NOTCH1 mutations on survival in paediatric T-cell leukaemia.

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

We are re-submitting the new version of the manuscript with the answers addressed to us by the reviewers. All modifications in the manuscript are highlighted. We have also included two supplementary documents as additional files.

#Ref 1. Claudia Baldus

Major:

Q1. The mutational analysis of FBXW7 should also be included in the experimental set up.
A1. FBXW7 mutations (ex9-10) were screened in 110 T-ALL DNA samples available and the final analysis of this marker alone or in combination with NOTCH1 is discussed all along the new version of the manuscript.

Q2. The authors do not provide any association between the NOTCH1 mutation status (complex vs. point mutation) and other clinical as well as molecular variables (including, age, risk group, CD1a expression…)
A2. Yes, we did provided the statistical analysis that showed association between NOTCH1 type of mutations and clinical-molecular data in Table 2.

Q3. The prognostic impact is only related to overall survival, an endpoint that is highly influenced by treatment related or other non-leukemia related events. The authors should provide other endpoints including the CR rate, relapse rate, MRD and DFS to illustrate that the proposed poor outcome is related the recurrence of the leukemia rather than treatment associated events.
A3. We added new statistical analysis regarding other survival endpoints as clinical CR and event free-survival. Unfortunately, the MRD was not uniformly performed in different Brazilian hospitals that sent samples to our studies, so this endpoint could not be evaluated here.

Q4. The authors will have to be more precise on the multivariable model: how was this constructed, what variables were included in the model, etc. Was expression of CD1a included in the model?
A4. No CD1a expression was not included, because the inclusion criteria used for multivariate analysis was only regarding the variables associated to a \( p \geq 0.1 \) in univariate analysis such as the type of NOTCH1 mutations (point vs. complex mutations), SIL-TAL1 fusion and induction responses.
Q5. It is difficult to understand why complex mutations should impact differently on outcome than point mutations with respect to treatment response as hypothesis by the author (page 12). Is there experimental evidence that would support these findings?

A5. We found that complex mutations were associated with a longer survival time than point mutations (p=0.031), although the impact of mutation type did not stand as a prognostic factor for EFS. We believe that this lack of significance in the EFS analysis could be due to the small number of patients (n=38) with EFS and NOTCH1 type of mutations available data. Then, we indicate the need to replicate this finding in an independent group of patients. Given the distinct functional effects of different mutations in NOTCH1 domains, our results raises the issue of the potential differential role of NOTCH1 mutation in the course of disease. Although no functional study has addressed this issue, we speculate that complex mutations would alter the Notch1 protein structure more drastically than point mutations, so blast cells would be more vulnerable to therapy responses. It is also possible that, as complex mutations were seen associated with high WBC, cases with complex mutations their presence lead to an initial more aggressive treatment, and hence to a sustained survival.

Q6. The authors claim (page 12) that SIL-TAL1 is of independent adverse prognostic impact, but since there is no multivariable analyses performed to demonstrate these finding…

A6. As mentioned before, Multivariate Cox analysis was performed including variables associated to a \( p \leq 0.1 \) in univariate analysis such as the type of NOTCH1 mutations (point vs. complex mutations), SIL-TAL1 fusion and induction responses. This analysis identified SIL-TAL1 (HR 3.12; 95% CI 1.48-6.57) and induction failure (HR 3.07; 95% CI 1.44-6.55) as independent negative prognostic factors.

Q7. As in the title the authors conclude at the end of the conclusion that complex NOTCH1 mutations confer a favourable outcome…

A7. The title and the conclusion were rephrased as requested.

Q8. Abstract: in contrast to the conclusion in the text, the authors claim in the final sentence of the abstract that the NOTCH1 mutations should be tested for further treatment strategies. However without a clear prognostic and predictive impact and without targeted therapies available this statement cannot be accepted this way.

A8. Due to all new survival statistical analysis, including the FBXW7 and other survival endpoints, the abstract was partially modified, as well as, the conclusion.

Minor:

Q9. The number of patients for each group has to be indicated in all of the figures.

A9. The number of patients was indicated in each figure legend.
Minor Comments:

Q1. The genomic DNA extraction methods are included within the paragraph NOTCH1 mutations. It would be better to describe this in a separate paragraph. Apparently 3 different extraction methods were used, but it is not apparent which and how many samples were prepared by this different extraction methods.
A1. Ok, a new section was included in the manuscript Methods.

Q2. Was a FICOLL separation performed in advance, what was the percentage of blasts within the samples considered adequate for analysis.
A2. Explanation regarding FICOLL separation was included in methods section as well as the blast percent criteria for proper analysis.

Q3. The definition of the type “complex mutation” is confusing and should be replaced by a more precise definition.
A3. A more precise definition was included in Methods section.

Major Comments:

Q4. The authors state, that they want to perform a NOTCH pathway analysis. If they do not replace this term, it would be necessary to have data on the FBXW7 status. FBXW7 is mutated in approximately 20% of patients with mutated NOTCH.
A4. FBXW7 mutations (ex9-10) were screened in 110 T-ALL DNA samples available and the final analysis of this marker alone or in combination with NOTCH1 is discussed all along the new version of the manuscript.

Q5. The authors wanted to demonstrate the significance of each type of NOTCH1 Mutation regarding disease recurrence (p.3). If the authors really want to answer this question, an analysis of initial response, the disease-free survival, event-free survival and remission duration in relation to the molecular analysis should be provided.
A5. We added new statistical analysis regarding other survival endpoints as clinical CR and event free-survival. Unfortunately, the MRD was not uniformly performed in different Brazilian hospitals that sent samples to our studies, so this endpoint could not be evaluated here.

Q6. The discussion and conclusion is somewhat superficial, e.g. there should be a paragraph in which the authors compare their results with the literature added for the SIL-TAL1 results.
A6. The discussion was almost completed modified adding more clearly differences between our own data and the literature.