Author's response to reviews

Title: Myeloid antigens in childhood lymphoblastic leukemia: clinical data point to regulation of CD66c distinct from other myeloid antigens

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Author's response to reviews: see over
Reviewer's report (1)
Myeloid antigens in childhood lymphoblastic leukemia: clinical data point to regulation of Title:
CD66c distinct from other myeloid antigens
1 4 January 2005 Version: Date:
Frederick Behm Reviewer:
Reviewer's report:
General
After almost two decades the mechanism of aberrant expression of myeloid-associated antigens by
blasts of precursor B-cell acute lymphoblastic leukemia (B-ALL) remains elusive. Although aberrant
myeloid antigen expression in ALL is not predictive of treatment outcome, it has proven valuable in
monitoring minimal residual disease and in a minority of B-ALLs is predictive of several nonrandom
chromosomal abnormalities. In a well-designed study, the authors' data suggest that the mechanism
of CD66c expression by B-ALL differs from other myeloid antigens including CD13 and CD33. Their
study further shows that CD66c expression, unlike CD13 and CD33, is stable from diagnosis to
relapse. Although the number of cases included in this study is relatively small and the clinical
follow-up interval short, this study is in line with other reports that CD66c expression by B-ALL is not
prognostically significant.

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Major Compulsory Revisions (that the author must respond to before a decision on publication can
be reached)

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Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the
author can be trusted to correct)

1. Authors should explain why a 20% value for a positive CD66c antigen expression was chosen as
listed in Table 1 & Figure 1 but not detailed in the Methods section. The authors state that values of
5%, 10%, and 50% were looked at for prognostic significance (Results, Prognostic significance of
CD66c expression subsection).

Added to Methods section: Value of 20% was chosen as a threshold of positivity as recommended by EGIL {Bene, 1995
#314}. For robust prognostic significance testing, other threshold values were also tested as indicated in results.

Were various cut off values for positivity similarly looked at when comparing the frequency of expression
of CD13, CD33, and other myeloid antigens with CD66c?

The analysis was extended to different cutoff values and the description is added to Par. Frequency of CD66c and
myeloid antigen (MyAg) expression in the Results.

2. The "...known risk factors..." examined in conjunction with CD66c expression (Results, Prognostic
significance of CD66c expression subsection) should be listed, preferably in Table format.

Citation to BFM-95 stratification added to the Results, summarized in a new Table 2.

3. The total number of CD66c positive cases in Figure 1 is 41% (150 of 365 cases) but is listed as
43% in the Results, first paragraph.

Mistake found in Figure 1, figure repaired.

4. The unit for the Y axis for Figure 3 needs clarification.

A sentence added to figure legends:
CEACAM6n value is normalized to beta-2-microglobulin (see Methods)

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Discretionary Revisions (which the author can choose to ignore)
Reviewer's report (2)
Myeloid antigens in childhood lymphoblastic leukemia: clinical data point to regulation of Title: CD66c distinct from other myeloid antigens
1 18 January 2005 Version: Date:
Vincent H van der Velden Reviewer:
Reviewer's report:
General
The authors analyzed 365 precursor-B-ALL patients and show that (1) CD66c (KOR-SA4544) is expressed on 43% of cases, (2) CD66c expression is negatively correlated with the expression of other myeloid antigens (CD13, CD33, and CD65), (3) CD66c expression is stable between diagnosis and relapse, and (4) CD66c expression has no prognostic relevance. In addition, the authors show, in contrast to previously published data, that CD66c is invariably expressed on the cell surface and is not retained in the cytoplasm.
The experiments seem to be performed well and the manuscript is clearly written.

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Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)
- The authors performed flowcytometry to determine the intracellular expression of CD66c. It is however not clear how this experiment was exactly performed and what data are shown in figure 2. It now seems that cells were either immediately used for membrane staining with CD66c, or were first permeabilized, followed by (membrane and intracellular) CD66c staining (in combination with other markers for gating?). Theoretically, all samples should at least be on the 45° angle line (or above), but in figure 2 several cases have a lower percentage of CD66c positive cells if CD66c is determined on permeabilized cells as compared to cells with CD66c membrane staining only. The experiment would have been more elegant if, for example, membrane-bound CD66c was first stained using the KOR-SA3544 antibody, followed by permeabilization and subsequent staining with the 9A6 antibody (labeled with a different fluorochrome).
Cells were fixed, permeabilized and then stained with KOR-SA3544–FITC. This approach sums the signals from cytoplasm and from surface. In the same time, background fluorescence increases. Borderline events thus may become falsely negative, as now mentioned in Results. In neutrophils, which store the CD66c molecules in granules, the signal after fixation and permeabilization markedly increases. Thus the cytoplasmic staining is quantitatively less sensitive than surface staining but sensitive enough to reveal intracellular localization. The prime reason for this experiment was to check whether CD66c\textsubscript{[surface neg]} cells store the molecules intracellularly – the issue of summing the surface and intracellular signal was not prominent here.

- The authors indicate that CD66c negative cells of heterogeneous populations showed higher CD66c transcript levels than CD66 negative cells from homogeneous populations. However, since the transcript level of CD66c in CD66c positive cells is about 100-times higher, minor contaminations (around 1%) will already result in such seemingly different transcript levels. In my opinion it is more likely that the observed difference is due to such minor contaminations.
Positive transcript may come from either low basal production by surface negative cells or from a minor contamination as suggested. We cannot formally rule out a contamination of the sorted specimen by positive cells due to small
numbers of cells, as we had to opt for sorting directly into the RNA isolation solution (Trizol). Generally, we see sorting contamination in this setup of about 1% but not in the order of 10%. There is only one sample with positive signal in CD66c<sup>neg</sup> fraction that differs by 100 fold from CD66c<sup>pos</sup> fraction and can be caused by contamination, three other cases differ by 10 fold or less which is less like to be caused only by contamination. In any case, the main point of this experiment is to show that CD66c<sup>neg</sup> cases do not contain comparable amount of CEACAM6 transcript as would be expected if all cases would generate the protein as suggested by Sugita.

Furthermore, it would be interesting to see whether there is a correlation between CD66c protein expression levels and CD66c transcript levels, both determined on the total leukemic population. The correlation of surface protein with transcript level is only weak, as shown in the fig. here. (Dashed lines mark out the 95% confidence interval, regression with a coefficient is shown.) We do not intend to include it in the paper at present.

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Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

- Material and Methods, section ‘Patients’: the authors should indicate in how many patients TEL/AML1, BCR/ABL or MLL/AF4 fusion gene transcripts were detected.
  Shown in additional Figure 1

- Although reported before, it may be interesting for the readership of BMC to briefly indicate the correlation between CD66c expression and genotype (presence of particular fusion gene transcript, hyperdiploidy) in the current patient series.
  Shown in additional Figure 1

- The authors should not refer to submitted data (reference 26), but should include this in the discussion as ‘unpublished results’.
  Changed as suggested

- Legend Figure 1: ‘CD66c positivity excludes positivityâ€’ is not correct, as some cases do express both CD66c and another myeloid antigen.
- Figure 6: Instead of showing the total number of patients in each graph, it would be easier to indicate for each graph the number of patients in the CD66c positive and CD66c negative group. The BCR-ABL positive group is too small for statistical analysis; this figure can be deleted and results can be mentioned briefly in the text. Also results in the MLL-AF4 positive group can be mentioned in the text briefly.

Figure changed as suggested, prognostic significance testing summarized in a new Table 2

Discretionary Revisions (which the author can choose to ignore)
- CD66c expression is, in many cases, only expressed on a subset of the leukemic blast cells. This implies that, when applied for MRD analysis, CD66c will only detect a part of the leukemic cells. The authors may discuss the use of CD66c for MRD analysis in some more detail in the 'Discussion'. We agree that this area is very important but we did not feel to discuss the issue beyond what is substantiated by the presented data. The sensitivity and specificity of CD66c at MRD should be tested by correlation with clinical data and/or independent techniques such as immunoreceptor gene translocations and fusion transcript PCR.

What next?:
Unable to decide on acceptance or rejection until the authors have responded to the major compulsory revisions

Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Acceptable
Statistical review: No
Declaration of competing interests:
I declare that I have no competing interests