Author's response to reviews

Title: Flow-cytometric monitoring of disease-associated expression of 9-O-acetylated sialoglycoproteins in combination with known CD antigens, as an index for MRD in children with acute lymphoblastic leukaemia: a two-year longitudinal follow-up study

Authors:

Suchandra Chowdhury (cmandal@iicb.res.in)
Suman Bandyopadhyay (cmandal@iicb.res.in)
Chandan Mandal (cmandal@iicb.res.in)
Sarmila Chandra (cmandal@iicb.res.in)
Chitra Mandal (cmandal@iicb.res.in)

Version: 2 Date: 26 November 2007

Author's response to reviews: see over
To,
The Editor, BMC Cancer, Attention to Dr. Mona Jazayeri, Assistant editor,
BMC-series journals,
BioMed Central Ltd, Middlesex House, 34-42 Cleveland Street, London W1T 4LB, UK
Tel: +44 (0)20 7631 9921
Facsimile: +44 (0)20 7631 9923
e-mail: editorial@biomedcentral.com
Web: http://www.biomedcentral.com/

Sub: Submission of newly REVISED manuscript entitled “Flow-cytometric monitoring of
disease-associated expression of O-acetylated sialoglycoproteins in combination with known
CD antigens, as an index of MRD in children with acute lymphoblastic leukaemia: a two-year
longitudinal follow-up study” for consideration as a “Research article”.

Ref: MS: 3806764931566294

Dear Dr. Mona Jazayeri,

Let me take this opportunity to thank you for all the valuable comments sent by the reviewers.
We have tried our level best to answer all the comments by reviewer 1- Prof. Giuseppe Gaipa to
improve the Ms. Now we are submitting the newly revised manuscript titled “Flow-cytometric
monitoring of disease-associated expression of O-acetylated sialoglycoproteins in combination with
known CD antigens, as an index of MRD in children with acute lymphoblastic leukaemia: a two-year
longitudinal follow-up study” for kind consideration in your journal as a ‘Research article’.

We have modified the manuscript giving a point-by-point response to the concerns raised by
Prof. Giuseppe Gaipa. Please note, we have submitted two colour figures for better representation
(only for Prof. Giuseppe Gaipa). The same figures are already there in the Ms in black and white. We
are attaching the answers to the comments along with the new figures at the end of this letter.

This work has not been published and is not being considered for publication elsewhere. On
behalf of all the authors, this is to state that all named authors have agreed to the submission and
have participated in this study to a sufficient extent to be named as authors.

We believe that this newly revised manuscript will find a place in your esteemed journal for
publication without further delay.

With best regards,

Yours sincerely,

Dr. Chitra Mandal
Answers to the reviewers comments

Reviewer 1

Title: Flow-cytometric monitoring of disease-associated expression of 9-O-acetylated sialoglycoproteins in combination with known CD antigens, as an index for MRD in children with acute lymphoblastic leukaemia: a two year longitudinal follow-up study

Version: 1 Date: 26 September 2007

Reviewer: Giuseppe Gaipa

Reviewer's report:

General

The manuscript has been improved. Nevertheless some questions remain opened.

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

Answer to Q1: The threshold of positivity to distinguishing between normal lymphocytes and leukemic blasts is crucial. The terms "minimal presence" and "higher expression" cannot be used alone as an objective quantitative criteria in MRD detection. Numbers have to be indicated. The use of CD45 is not included in the MRD probes, then its contribution in addition to the others markers in the detection of MRD during follow-up is hardly evaluable. We think it should not be mentioned in this context.

Answer- We also strongly believe that distinguishing between normal lymphocytes and leukemic blasts is crucial for this study. We have tried our best to highlight this point by introducing a colour figure (pl. see below).

The normal lymphocytes express 5-10% OAcSGP, MFI being 10-50au (Figure 1e, see below), using single colour FACS analysis with FITC-Achatinin-H. In contrast, under identical condition, diagnostic blasts have 40-90% of OAcSGP+ cells, MFI being ~100-700 au (Figure 1b, see below) using only FITC-Achatinin-H.

The differences between MFI, thus, emphasizes the over expression as well as higher antigen density of OAcSGP on the cell surface of the cancerous blasts as compared to the normal lymphocytes. This has been one of the major distinguishing features between the two.

The following new figure (Figure 1) represents the differential expression of OAcSGP with other CD antigens using triple colour FACS Analysis (for reviewer only). A similar figure is already provided in black and white as Fig 2 in the revised MS.
**Figure 1:** Differential distribution of OAcSGP$^+$ cells using triple colour FACS analysis to distinguish between cancerous and normal cells

**Figure 1a:** Mononuclear cell population (MNCs, BLACK dots), gated for the lymphoblasts (R1, BLACK dots), from a diagnostic B-ALL patient.

**Figure 1b:** MNCs in R1 are assessed for the status of OAcSGP (by binding with Achatinin-H) and gated as R2. For simplicity, here the R2 gating is subdivided such that the OAcSGP$^{lo}$ cells are gated as R2$^1$, shown in RED while the OAcSGP$^{hi}$ cells are gated as R2$^2$, shown in BLUE.

**Figure 1c:** Dot plot with CD10 versus CD19 representing the R2$^1$ and R2$^2$ populations. The cells in R2$^1$ (OAcSGP$^{lo}$) are found to be CD10$^{-}$CD19$^{lo}$ while those in R2$^1$ (OAcSGP$^{hi}$) are CD10$^{hi}$CD19$^{hi}$ occupying the R3 gate. The R3 gate is the region set for the presence of cancerous blasts and for MRD in patients at clinical remission.

**Figure 1d:** Mononuclear cell population (MNCs, BLACK dots) gated as R1 (GREEN dots) from a representative normal individual.

**Figure 1e:** MNCs in R1 are assessed similarly (like Figure 1b) for the status of OAcSGP (by binding with Achatinin-H) under identical condition. Only the OAcSGP$^{lo}$ cells are present, gated as R2, shown in RED.
Figure 1f: Dot plot with CD10 versus CD19 representing the R2 population. The cells in R2 (OAcSGP\textsuperscript{lo}) are all found to be CD10\textsuperscript{−}CD19\textsuperscript{lo}. The R3 gate set is devoid of any cell. Thereby, we may conclude that the OAcSGP\textsuperscript{lo} cells, shown in RED dots, show scatter similar to normal lymphocytes and there is complete absence of OAcSGP\textsuperscript{hi} cells. In contrast, both OAcSGP\textsuperscript{hi} and OAcSGP\textsuperscript{lo} populations are found in patients, at diagnosis.

Apart from these criteria, the templates that we have used for MRD detection, being OAcSGP\textsuperscript{+}CD10\textsuperscript{+}CD19\textsuperscript{+} or OAcSGP\textsuperscript{+}CD34\textsuperscript{+}CD19\textsuperscript{+} for B-ALL and OAcSGP\textsuperscript{+}CD7\textsuperscript{+}CD3\textsuperscript{−} for T-ALL is ALL specific i.e., the R3 region in normal donors or in patients with other haematological malignancies have no cells.

To reconfirm our distinction between normal lymphocytes and cancerous blasts and to answer the reviewer’s comment, we mentioned about CD45 in the previous ‘answer to the comments’. However, as it is not mentioned in detail in the Ms, as per the reviewer’s comment we are omitting that portion.

2) Answer to Q2:CD10 and CD34 are expressed in bone marrow lymphocytes, in particular in regenerating samples. This criteria can not be adopted to distinguish between normal and leukemic B cells. Only the differential expression of these markers can be adopted for the purpose.

Answer- Yes, we fully agree with the fact that CD10 and CD34 are expressed in normal bone marrow lymphocytes, in particular in regenerating samples, therefore, their presence and absence can not be adopted to distinguish between normal and leukemic B cells.

Therefore, we have used the differential expression of CD10 and CD34 for the MRD study. The new figure 2 (in colour, shown in black and white as Fig 1 in revised Ms) is suggestive of the same.
Figure 2: Differential distribution of CD10 in MNCs of a diagnostic B-ALL patient using triple colour FACS analysis to distinguish between cancerous and normal cells

**Figure 2a**: Mononuclear cell population (MNCs, BLACK dots), gated for the lymphoblasts (R1, RED dots), from a diagnostic B-ALL patient.

**Figure 2b**: MNCs in R1 are assessed for the status of CD10. CD10$^+$ cells are gated as R2, shown in BLUE. The CD10$^{lo}$ or CD10$^-$ fractions are shown in RED, outside R2 quadrant.

**Figure 2c**: Dot plot with Achatinin-H versus CD19 showing the differential distribution of CD10 shown in Figure 2b

The CD10$^{hi}$ cells (BLUE dots in R2) are found in R3 gate being OAcSGP$^{hi}$CD19$^{hi}$ population, considered to be lymphoblasts in B-ALL. In contrast, the CD10$^{lo}$ or CD10$^-$ fractions (RED dots, outside R2 quadrant, figure 2b), while gated for R3 (Figure 2c) showed scatter outside R3 region, thereby, considered to be normal lymphocytes. Therefore, two populations exist in patient, representing OAcSGP$^{hi}$CD19$^{hi}$CD10$^{hi}$ and OAcSGP$^{lo/}$CD19$^{lo/}$CD10$^{lo/}$, thus confirming the differential distribution of CD10.

Similar observation was also found with CD34$^+$ cells having both OAcSGP$^{hi}$CD19$^{hi}$CD34$^{hi}$ and OAcSGP$^{lo/}$CD19$^{lo/}$CD34$^{lo/}$ populations.

3) Answer to Q4: The question How many patients (not samples) per time point are MRD+?, seems still open. We could not appreciate this information from table 1.
Answer- In the Ms a range of MRD (0.04-0.06%) has been proposed for patients in clinical remission, during the two years study. In this two-year study, patients in clinical remission had MRD within this range. However, children with higher this value were susceptible to relapse (Fig 5, 6).

A few children, within this range i.e. 0.04-0.06%, when monitored beyond two years was MRD negative (10 samples). However, the fact that they had a minimal MRD during the two years of treatment was probably because they were still receiving treatment.

Please note that, the major aim of this manuscript was to establish a template to increase the sensitivity for the detection of MRD exploring the presence of OAcSGP along with known lineage specific markers. More importantly, compare the differential expression of these antigens, used for detection of MRD, in paired sample of PB and BM from the same patients for possible future use of only PB instead of BM even in B-ALL patients. Interestingly, we are able to predict relapse even in PB using these templates. We are still continuing with the study beyond two years of treatment.

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

Discretionary Revisions (which the author can choose to ignore)

The title of the paper should be more concerned to the feasibility of the proposed flow cytometric approach rather than the longitudinal follow-up, as the clinical impact of data presented have to be validated during longer observation time.

What next?: Accept after minor essential revisions

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Needs some language corrections before being published

Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.

Declaration of competing interests: I have no competing interests.
Reviewer 2

Title: Flow-cytometric monitoring of disease-associated expression of 9-O-acetylated sialoglycoproteins in combination with known CD antigens, as an index for MRD in children with acute lymphoblastic leukaemia: a two-year longitudinal follow-up study

Version: 1 Date: 27 September 2007
Reviewer: Anthony Corfield

Reviewer's report:

General.
The revisions made to the paper have addressed all of the points raised earlier.
There are no additional issues to raise

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

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

Discretionary Revisions (which the author can choose to ignore)

What next?: Accept without revision

Level of interest: An article of importance in its field

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests: I declare that I have no competing interests
Reviewer 3

Title: Flow-cytometric monitoring of disease-associated expression of 9-O-acetylated sialoglycoproteins in combination with known CD antigens, as an index for MRD in children with acute lymphoblastic leukaemia: a two-year longitudinal follow-up study

Version: 1 Date: 19 November 2007

Reviewer: Seth J Corey

Reviewer’s report:

I have reviewed the revision, the responses by the two reviewers, the original critique of reviewer 3, and the response of the authors to the critiques. Besides the need to correct the syntax and reduce the length of the manuscript (especially the introduction and discussion), the manuscript should be revised along the suggestions of Dr. Gaipa. Following revisions according to her critique, this paper should be acceptable and provide a new target for MRD detection.

Answer- Thank you for the comment. We have made all the necessary changes as per the reviewers’ comment.