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Original Article

Rituximab added to standard chemotherapy and its effect on minimal residual disease during induction in CD20 positive pediatric acute lymphoblastic leukemia: a pilot RCT

Aditya Kumar Gupta[1], Anita Chopra[2], Jagdish Prasad Meena[1], Jay Singh[2], Ravindra Mohan Pandey[3], Sameer Bakhshi[4], Rachna Seth[1]

[1]Division of Pediatric Oncology, Department of Pediatrics, All India Institute of Medical Sciences, New Delhi 110029, India; [2]Department of Laboratory Oncology, Institute Rotary Cancer Centre, All India Institute of Medical Sciences, New Delhi 110029, India; [3]Department of Biostatistics, All India Institute of Medical Sciences, New Delhi 110029, India; [4]Department of Medical Oncology, Institute Rotary Cancer Centre, All India Institute of Medical Sciences, New Delhi 110029, India

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Abstract: The use of rituximab in the treatment of pediatric acute lymphoblastic leukemia (ALL) has been evaluated but mostly this has been done in the setting of a relapsed or refractory disease. Addition of rituximab to the initial treatment regimen improves the outcomes in adult CD20 positive ALL. This study was done to study its effect on newly diagnosed CD20 positive pediatric ALL patients. Twenty pediatric patients with CD20 positive ALL were randomly assigned to receive rituximab along with standard-chemotherapy [Intervention-arm (IA)] or standard-chemotherapy alone [Standard-arm (SA)]. The absolute blast count (ABC) on day 8, flowcytometry-MRD levels in the peripheral blood (PB) on day-8, day-15 and in the bone marrow (BM) at end of induction (EOI) were the outcome variables. Baseline characteristics were comparable between the IA (n=10) and SA (n=10). Significantly lower day-8 ABC was seen in the IA (P=0.005). The day-8 PB-MRD showed lower values for the IA but the difference wasn't significant (P=0.22). There was no difference between the IA and SA for day-15 PB-MRD and EOI BM-MRD. There was no difference in the incidence of adverse effects. Rituximab added to standard-chemotherapy lead to lower day-8 ABC and lower day-8 PB-MRD in CD20 positive pediatric ALL patients. Rituximab may be beneficial in pediatric ALL treatment. Studies with larger sample size are needed for more evidence.

Keywords: CD20, rituximab, pediatric acute lymphoblastic leukemia

Introduction

Improvement in the outcome of pediatric ALL patients in the recent past has been attributed to risk stratified administration of intensive chemotherapeutic regimens. The use of intensive chemotherapy is associated with myelosuppression and other side-effects. In developing countries these side effects can cause deaths in approximately 10% of patients. About 60% of these deaths can occur in the first phase of treatment itself [1]. The proportion of deaths due to side effects of chemotherapy is more in developing countries.

Monoclonal antibodies directed against specific antigens that the cancer cells may express, are now being incorporated into the treatment of many cancers. Monoclonal antibodies are specific for a particular antigen and cell type and do not have the generalised toxicity encountered with conventional chemotherapy. In high-risk situations like refractory or relapsed malignancies these agents have been used with considerable success.

CD20 expression (defined as expression in greater than 20% of the leukemic cells by flowcytometry) is found in about 40-50% of precursor B-cell ALL and in approximately 90% of mature B-cell ALL [2]. The prognostic significance of CD20 expression in pediatric ALL is still unclear, but some studies suggest that these patients do not do as well as the CD20

negative ones [3]. CD20 expression in adult ALL has been associated with sub-optimal outcomes. Rituximab is a monoclonal antibody directed against CD20 and its incorporation into the chemotherapeutic regimens of CD20 positive malignancies such as Burkitt's lymphoma and Hodgkin lymphoma is associated with better outcomes. The poor prognosis of CD20 expression in adults with B-cell precursor ALL, prompted the incorporation of rituximab into chemotherapy regimens for adult ALL. Few studies provide evidence that adding rituximab to chemotherapy improves the outcomes for adult patients with newly diagnosed CD20 positive ALL [4-6].

There are reports of the use of rituximab in pediatric ALL, but these are mostly in the setting of a relapsed or refractory ALL and it has not been evaluated in a clinical trial in the upfront treatment of pediatric CD20 positive ALL. This study was a pilot randomised trial conducted to prospectively test the effect of addition of rituximab to standard chemotherapy in CD20 positive pediatric ALL patients in the first phase of chemotherapy (induction phase of chemotherapy).

Methods

Study design

The study was a randomised controlled pilot conducted to generate preliminary data on the effect of rituximab on induction in pediatric CD20 positive ALL. The study was carried out in the Division of Pediatric Oncology (Department of Pediatrics) of the All-India Institute of Medical Sciences. The study was approved by the ethics committee of the institute and was registered prospectively with the Clinical Trials Registry of India [CTRI/2017/12/010897]. The study period was from 1st March 2018 to 16th January 2019.

Study population

The study was done in pediatric patients diagnosed with ALL. The patients were approached for enrolment in the trial when a diagnosis of ALL was made. The inclusion criteria were as follows:

- Patients with B cell ALL confirmed by flow-cytometry.

- Age less than 18 y.

- CD20 expression on the blasts by flow-cytometry.

The exclusion criteria were:

- Children who had received any form of chemotherapy or steroids before enrolment.

- CD20 expression on less than 20% blasts.

- Mixed phenotype acute leukemia.

- Relapsed ALL.

As it was a pilot study, 20 patients were enrolled, and the enrolment was stopped when the last patient (20th patient) entered the trial.

Method of randomisation and allocation concealment

The random sequence was generated by a statistician, not involved in the trial and it was kept in opaque sealed envelopes. Once an informed consent was obtained, the patients were randomised to either the intervention arm (IA) or the standard arm (SA) by opening the sealed envelopes serially.

Blinding

The study participants and clinical investigator (AKG, JPM, RS, SB) were not blinded. The investigators in charge of the laboratory evaluations (AC and JS) and the statistician (RMP) were blinded.

Treatment

The patients allocated to the standard arm (SA) as well as intervention arm (IA) got chemotherapy as per the risk group of their disease, based on the standard protocol for ALL followed in our unit (detailed below). In the IA the patients got an additional dose of rituximab as an infusion at 375 mg/m^2 either on day 1 or day 2 of starting of treatment. Before rituximab each patient got paracetamol and anti-histaminics as premedication. No additional doses of corticosteroids were given with rituximab infusion.

Patients were classified into standard, intermediate and high risk groups. The children who were standard risk (SR) were between 1 and 10 years of age, were prednisolone good respond-

ers (<1000 blasts/cumm of peripheral blood after 7 days of steroid treatment), had no high risk cytogenetics/molecular genetics, had no central nervous system (CNS) disease and had an initial total leucocyte count (TLC) less than 50,000/cumm. All patients who were prednisolone poor responders or had high risk cytogenetics/molecular genetics or had CNS disease were classified as high risk (HR). Patients who were <1 years or >10 years in age or had initial TLC >50,000/cumm or had bulky disease (lymph node mass >5 cm or liver/spleen reaching beyond umbilicus) were classified as intermediate risk (IR) in the absence of any of the high risk features.

The induction chemotherapy consisted of the following doses of anticancer drugs: Prednisolone given orally at dose of 60 mg/m^2; intravenous (IV) vincristine at 1.5 mg/m^2/dose; intramuscular (IM) L-asparaginase at 10000 IU/m^2/dose; IV daunorubicin 25 mg/m^2/dose and intrathecal methotrexate (8 mg for 1-2 y; 10 mg for 2-3 y and 12 mg for >12 y).

During the induction phase the following schedule for chemotherapy was followed: SR patients were given prednisolone for 28 days followed by tapering over 9 days, four doses of vincristine, four doses of L-asparaginase and three doses of intrathecal methotrexate. IR patients got prednisolone for 28 days followed by tapering, four doses of vincristine, eight doses of L-asparaginase, two doses of daunorubicin and three doses of intrathecal methotrexate. The HR patients were given prednisolone for 28 days followed by tapering, four doses of vincristine, eight doses of L-asparaginase, four doses of daunorubicin and three doses of intrathecal methotrexate.

Response to treatment

Measures for response assessment were absolute blast count (ABC) on day-8, peripheral blood (PB) and bone marrow (BM) minimal residual disease (MRD). The PB MRD was measured on day-8 and day-15. The BM MRD was measured at the end of induction (EOI) chemotherapy.

Multiparameter flowcytometry for immunophenotyping

BM samples were processed by ten-colour multiparameter flowcytometer for immunophenotyping using bulk lyse and stain method. The cell suspension from the BM aspirate was prepared by bulk erythrocyte lysing with ammonium chloride based lysing reagent. After lysis and wash, the remaining cells were resuspended in phosphate buffered saline (PBS) with 5% bovine serum albumin (BSA). The cells were then stained for immunophenotyping using 10-colour antibody panels.

All cells were fixed with 0.5% paraformaldehyde and stored at 4°C. The analysis was done within 6 hours of staining. Samples were acquired on a three-laser Gallios flowcytometry instrument (Beckman Coulter, BC). For the diagnostic immunophenotyping, 25,000 to 50,000 events per tube were acquired. Immunophenotyping data was analysed with Kaluza (version 1.3) software (Beckman Coulter, USA). The percentage of blasts expressing CD20 was noted in each case.

MRD studies

Minimal residual disease (MRD) is the measurement of the percentage of leukemic cells detectable by methods that are more sensitive and objective than morphologic examination. The detection of MRD by flowcytometry or polymerase chain reaction (PCR) based methods has become important in the prognostication and treatment stratification of leukemias. In this study for MRD analysis a minimum of 1,000,000 events were acquired in each case. MRD analysis was performed using the ten-colour antibody panel described above. Samples were labelled MRD positive based on the identification of a cluster of minimum 10 events with at least two leukemia associated immunophenotypes (LAIPs). MRD was calculated as a percentage of MRD positive events of the total nucleated cells. MRD value of greater than 0.01% was considered as positive.

Safety and monitoring

All adverse effects were reported to a data safety monitoring board that consisted of 3 experts of Pediatrics, who had a research and clinical experience of more than 10 years. All adverse effects were recorded as per the CTCAE version 3.0 [7].

Sample size and statistical methods

There was no available study on the efficacy of rituximab in reducing the MRD in children with

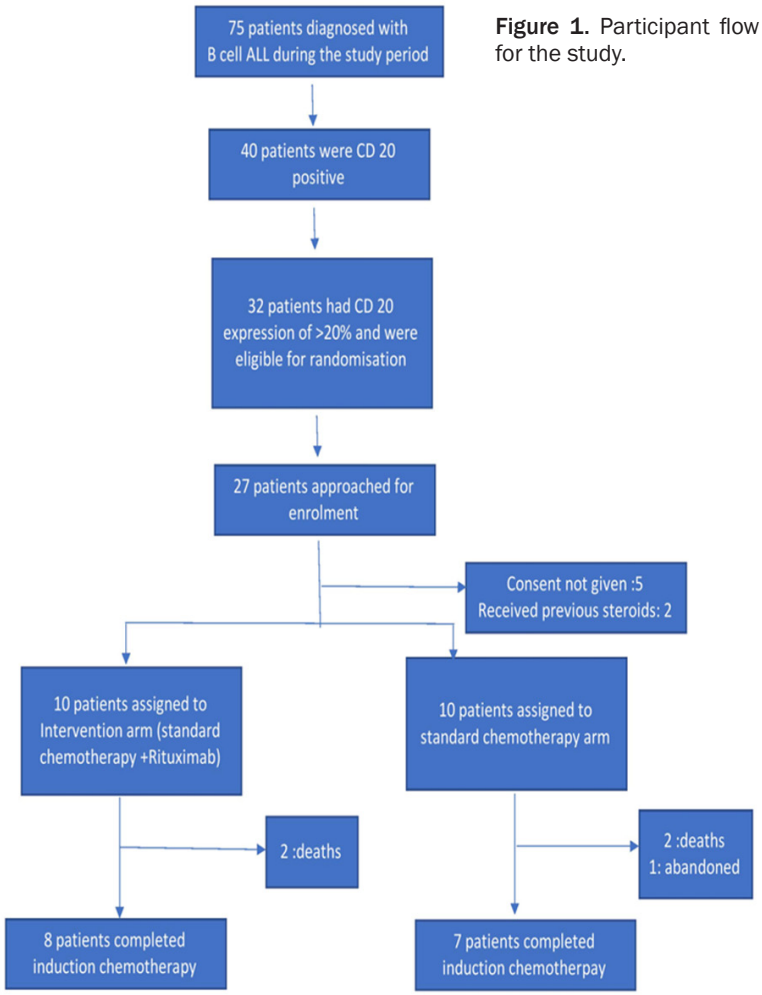

Figure 1. Participant flow for the study.

CD20 positive ALL. This study was done as a pilot study to generate evidence for the same. A sample size of twenty was chosen based on feasibility.

The categorical variables in the study are presented as percentage and continuous data as mean ± SD for normal distribution and as median with inter-quartile range for skewed data. The categorical variables were compared using chi-square test. The continuous data were compared using t-test for normally distributed data and Mann-Whitney U test for skewed data. Statistical analysis was carried out using the statistical package SPSS (SPSS version 16, for Windows). A *P*-value of less than 0.05 was considered statistically significant.

Results

Seventy-five patients were diagnosed with B cell ALL during the study period, out of which 40 patients were CD20 positive. CD20 expression in >20% blasts was found in 32 patients, who were eligible for the study. Informed consent was sought from 27 patients by the principal investigator (AKG). Of 27 patients, 2 had received steroids prior to presenting to our centre and were excluded. Consent couldn't be obtained in 5 patients. The flow has been depicted in **Figure 1**.

Baseline characteristics were comparable between the IA (n=10) and SA (n=10). Mean age for the group was 67 months. The SR:IR:HR ratio in both arms was 1:8:1. No patient had CNS disease. Cytogenetic studies were available for 15 patients, out of which abnormal cytogenetics were found in 5 participants (1:SA; 4:IA). In 5 patients the cytogenetic results could not be obtained due to failure of the experiment because of technical reasons. Molecular genetic studies were available for all patients and one patient in each arm had molecular aberrations that lead to re-classification of the patient as high risk [1 patient in IA positive for both MLL gene rearrangement and Philadelphia chromosome (Ph); 1 patient in SA was Ph positive]. The mean size of the liver and spleen at presentation were comparable between the two groups. The initial total leucocyte count, percentage of blasts in the peripheral blood and in the bone marrow in between the two groups were comparable. The expression of CD20 was found in 77.4±26.7% of BM blasts in the SA and in 58.2±31.7% in the IA. The difference was not significant. The expression of CD20 on the PB blasts was significantly more in the SA (87.7±23.4% vs 62.6±30.1%; P=0.04) (**Table 1**).

*Comparison at different time points of assessment between the two groups (**Figures 2, 3** and **Table 2**)*

(i) Day-8 assessment parameters [absolute blast count (ABC) and PB MRD].

At day-8, after 7 days of prednisolone (at 60 mg/m^2/day: both in IA and SA) and one dose of

Table 1. Baseline characteristics of participants in the standard arm and the intervention arm

	Standard Arm (n=10)	Intervention Arm (n=10)	*P*-value
Age (months)	54 (26-144)	57 (24-168)	0.70
Female:Male	1:9	2:8	0.53
Median Duration of illness (days)	52 (7-90)	31 (7-210)	0.54
Risk group of disease at initial evaluation			
Standard	1	1	NS
Intermediate	8	8	
High	1	1	
Median Initial TLC (Range) ($\times 10^3$/cumm)	61.5 (1.64-410)	14.4 (3.64-92.0)	0.13
CSF positivity	nil	nil	NS
Cytogenetics			0.34
Normal	7	3	
Abnormal	1	4	
Molecular genetic studies			0.36
Normal	9	9	
High risk	1	1	
Liver size (cm below right coastal margin)	4.65±1.97	4.55±1.92	NS
Spleen size (cm in its axis)	3.85±1.41	3.7±1.49	0.58
PB blasts (% of all WBC) on peripheral blood smear	64.9±32.2	46.8±21.7	NS
Blast % by flowcytometry in PB (of all cells counted)	68.4±16.9	40.5±7.4	0.99
CD20 positivity in PB (% of all blasts)	87.7±23.4	62.6±30.1	0.04
BM blasts (% of all WBC) on BM smear	91.6±6.61	87.5±20.4	0.71
Blast % by flowcytometry in BM (of all cells counted)	70.2±20.7	66.0±23.6	0.66
CD20 positivity in BM (% of all blasts)	77.4±26.7	58.2±31.7	0.92

PB: Peripheral blood; BM: Bone marrow; WBC: White blood cells; TLC: Total leucocyte count.

rituximab (at 375 mg/m^2: only in IA) the ABC in the peripheral blood and the PB MRD were assessed.

A significant difference was found between the two arms for day-8 ABC ($\times 10^3$/cumm) [0.46 (0.00-11.4): SA vs 0.00 (0.00-0.00): IA; p=0.005].

The mean day-8 PB MRD ($\times 10^{-4}$) [7.12 (0.00-36.6): SA vs 0.20 (0.00-39.9): IA; p=0.22] was lower for the IA but the difference did not reach the cut off for significance.

Four patients all in the SA, who were in the intermediate risk strata to start with, had a poor prednisolone response and their chemotherapy was upgraded to that of the high-risk category.

(ii) Day-15 PB MRD.

PB MRD values were available for 9 patients in each arm. There was no difference in the PB MRD values between the two groups.

(iii) End of induction BM MRD and attainment of MRD negativity.

End of induction MRD values were available for 7 patients in the SA and 8 patients in the IA. The median values of MRD were comparable in between the two groups however MRD positivity in the IA was in 2/8 patients compared to 1/7 in the SA.

Two patients (one in each arm) had induction failure and were counted as MRD positive. The other patient who had MRD positivity in the IA had missed treatment for 15 days after day-9 of chemotherapy due to unavoidable family circumstances. The above findings have been depicted in **Figure 2**.

*Adverse effects (**Table 2**)*

The adverse effects were graded as per the CTCAE v3.0. Seven patients in each arm had an adverse effect (P=0.68). There was no difference between the two groups amongst various grades of adverse effects. There were two

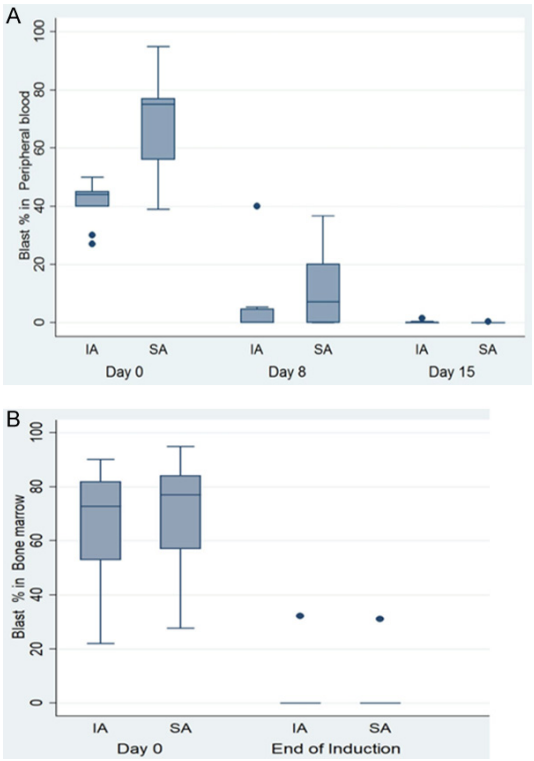

Figure 2. A. Box plot showing the percent of blasts by flowcytometry in the peripheral blood of the study participants at day 0 (before treatment), day 8 and day 15. B. Box plot showing the percent of blasts by flowcytometry in the bone marrow of the study participants at day 0 (before treatment) and end of induction.

deaths in each of the arms and one patient in the SA had to abandon treatment due to a progressive gangrene of the scrotum (**Table 2**).

Considering default, day-8 ABC >1000/cumm, abandonment, induction failure, positive MRD or death as events 50% of all participants experienced an event (4/10 in the IA vs 6/10 in the SA).

Discussion

Previous studies have shown that up to 48% of pediatric B cell ALL patients express the CD20 antigen [8]. The prognosis of the CD20 positive ALL patients has been shown to be poor in comparison to the CD20 negative ones by Borowitz et al. [3]. In our study, 32/75 (42.6%) of B cell ALL patients diagnosed during the study period were positive for CD20 (expression in >20% blasts). With the addition of rituximab to the chemotherapy in pediatric patients with CD20 positive B cell ALL, a significantly lower absolute blast count on day-8 of chemotherapy was achieved. The MRD levels in the peripheral blood were lower in the IA on day-8. Rituximab has been shown to be beneficial in adults with ALL. In a randomised trial involving 209 patients of ALL, from May 2006 to April 2014, Maury et al. demonstrated that addition of rituximab to the ALL chemotherapy, improved the outcomes in adults with CD20 positive, Philadelphia chromosome negative ALL (2 yr EFS of 65% vs 52% in controls). In the multivariate analysis, the rituximab arm had a longer EFS. The incidence of severe adverse effects and infections was similar in both groups [5]. Similarly, in 31 elderly patients with newly diagnosed Burkitt's Leukemia or B cell ALL, who received the hyper-fractionated cyclophosphamide, vincristine, doxorubicin, and dexamethasone (hyper-CVAD) regimen with rituximab, a superior outcome was achieved compared to a historical cohort that received hyper-CVAD alone [9]. The same group further studied two hundred eighty-two adolescents and adults with de novo Ph-negative precursor B-lineage ALL who were treated with standard or modified hyper-CVAD regimens. The later incorporated standard-dose rituximab if CD20 expression was >20%. It was found that the incorporation of rituximab into the hyper-CVAD regimen improved the outcomes for younger patients with CD20-positive Ph-negative precursor B ALL (Overall survival 75% vs 47% at 3 years, P=0.003) [4].

Rituximab exerts a direct toxic effect on the CD20 positive blasts. In the present study, evidence that rituximab may be effective in CD20 positive pediatric ALL can be derived from the fact that the absolute blast counts in the patients who received rituximab was lower on day-8. Four patients, all in the SA needed upgradation to high-risk group chemotherapy after day-8 due to their ABC being greater than 1000/cumm. Lower MRD values in the IA at day-8 further support the fact that rituximab was effective in achieving a deep remission in the CD20 positive patients. The benefit of rituximab in the IA that was present on D8 was not reflected on day 15 and at the end of induction. Four patients, all in the SA had their chemotherapy intensified because of suboptimal response on day-8 and this could be responsible for the day-15 PB MRD and EOI BM MRD

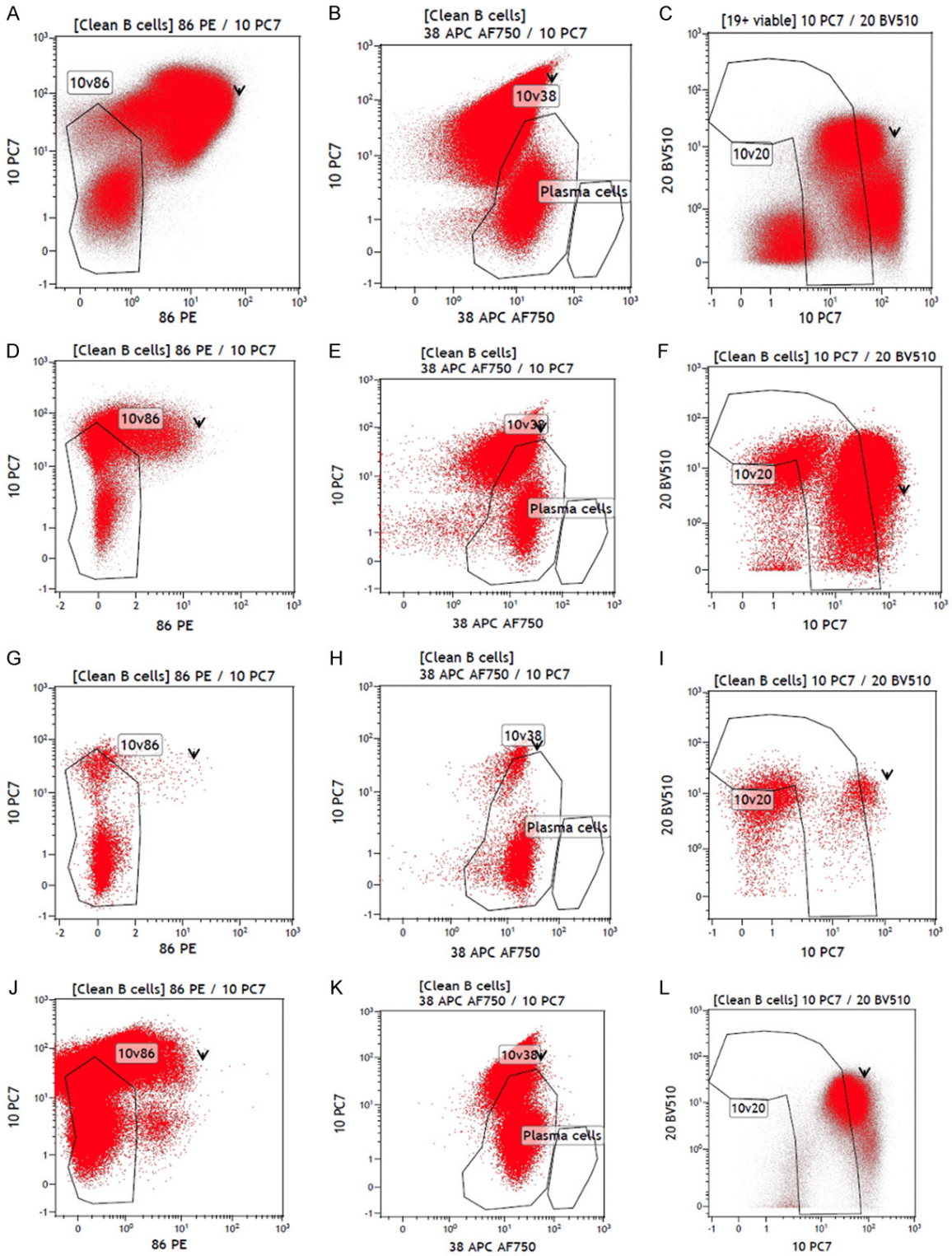

Figure 3. The figure shows CD19+ B cells pre-gated dot plots. A-C: At diagnosis. D-F: Day 8 peripheral blood. G-I: Day 15 peripheral blood. J-L: Day 30 bone marrow, (blasts marked by arrow).

values being comparable in the two arms. The sample size of 10 in each arm is small to make any generalisation.

In the present study only one dose of rituximab was given at the start of induction to assess its effect on the parameters of disease burden

Table 2. Comparison of disease assessment parameters and adverse effects during induction between the standard arm and the intervention arm

	Standard Arm (n=10)	Intervention arm (n=10)	*P*-value
Day 8 ABC ($\times 10^3$/cumm)	0.46 (0.00-11.4)	0.00 (0.00-0.00)	0.005
Day 8 PB MRD ($\times 10^{-4}$)	7.12 (0.00-36.6)	0.20 (0.00-39.9)	0.22
D 15 PB MRD ($\times 10^{-4}$)	n=9; 0.00 (0.00-0.42)	n=9; 0.00 (0.00-1.53)	0.51
EOI BM MRD ($\times 10^{-4}$)	n=7; 0.00 (0.00-31.1)	n=8; 0.00 (0.00-32.2)	0.37
MRD report	n=7	n=8	0.55
Negative	6	6	
Positive	1	2	
Adverse effects			0.68
Present	7	7	
Absent	3	3	
Grade of Adverse effect (CTCAE v3.0)			0.75
1	1	1	
2	3	2	
3	0	0	
4	1	2	
5	2	2	

PB: Peripheral blood; BM: Bone marrow; WBC: White blood cells; TLC: Total leucocyte count; ABC: Absolute blast count; MRD: Minimal residual disease; EOI: End of Induction; CTCAE: Common terminology criteria for adverse events.

during induction. Other investigators have used rituximab in CD20 positive adult ALL in varying schedules. In adult studies, repeated administration of rituximab reduces the incidence of relapse, without significantly affecting the rate or quality of complete remission. Prolonged administration is thought to play a role in sustained beneficial outcomes [5]. Whether the transient beneficial effect of rituximab on the day-8 evaluation parameters could be sustained in pediatric ALL by a more frequent administration, could be a question for research in future larger trials.

During the induction the probability of occurrence of adverse effects was similar in both the arms. There were two deaths in each arm. Maury et al. too found that addition of rituximab to chemotherapy did not significantly increase toxic effects or the cumulative incidence of death during the first remission in adult ALL patients [5]. In our cohort, two patients required stopping of the rituximab infusion due to infusion related reactions, and in both the infusion could be successfully completed after restarting at a slower rate. Other authors also have found that rituximab is well tolerated with the most common adverse events being infusion related, occurring during or shortly after the first infusion [10].

The present study has its limitations owing to a small sample size, a single dose of rituximab being used that is unlikely to affect long term outcomes and a short follow up of the patients. However, the present study provides evidence that rituximab has a beneficial effect on CD20 positive pediatric ALL, like it has in adult CD20 positive ALL. Larger studies are needed to support or refute the fact. In developing countries a significant contribution to the suboptimal outcomes in pediatric ALL, is due to the mortality and morbidity caused by the side effects of conventional chemotherapy. Monoclonal antibodies do not possess the generalised toxicity of conventional chemotherapy. Based on this small study, it is difficult to advocate substitution of toxic agents with rituximab/addition of rituximab to conventional chemotherapy, with the aim of decreasing side effects and improving survival. However, larger studies may be undertaken in future in a subset of ALL patients (e.g. relapsed CD20 positive ALL) and this strategy could be employed to generate stronger evidence.

Conclusions

Rituximab added to standard-chemotherapy was effective in achieving lower day-8 ABC and lower day-8 PB MRD in CD20 positive pediatric

ALL patients. Addition of rituximab didn't lead to a significant increase in adverse effects when compared to the standard chemotherapy. Studies with larger sample sizes are needed to further test its efficacy. Whether the benefit of rituximab seen on day-8 can be sustained by its administration in the later phases of chemotherapy, is a question that could be answered by future studies. Although previous studies exist where rituximab has been tested in pediatric ALL, but these are mostly in the settings of relapsed or refractory ALL. The novelty of our study is that it is one of the first studies in the pediatric setting where the use of rituximab has been tested in a randomised trial for the treatment of ALL upfront.

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Disclosure of conflict of interest

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Abbreviations

ALL, Acute lymphoblastic leukemia; CD, Cluster of differentiation; IA, Intervention arm; SA, Standard arm; IM, Intramuscular; IV, Intravenous; MRD, Minimal residual disease; PB, Peripheral blood; BM, Bone marrow; EOI, End of induction; LAIP, leukemia associated immunophenotypes.

Address correspondence to: Aditya Kumar Gupta, Division of Pediatric Oncology, Department of Pediatrics, All India Institute of Medical Sciences, New Delhi 110029, India. Tel: +91-7838379837; E-mail: adivick@gmail.com

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