CD9 as a Potential Prognostic Biomarker in Paediatric B-Cell Acute Lymphoblastic Leukaemia

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ABSTRACT

Objective: To evaluate the role of CD9 for predicting *ETV6::RUNX1*, *BCR::ABL1*, and *KMT2A* fusion genes with prognostic significance in B-cell acute lymphoblastic leukaemia (B-ALL).

Study Design: An observational study.

Place and Duration of the Study: Indus Hospital and Health Network, Karachi, Pakistan, from May 2020 to August 2022.

Methodology: Data of 488 paediatric (B-ALL) children diagnosed by flow cytometry were retrieved. Recurrent genetic abnormalities for *BCR::ABL1*, *ETV6::RUNX1*, and *KMT2A* fusion genes were retrospectively monitored. Fisher's Exact, Pearson's Chi-Square, and Mann-Whitney U tests along with univariate and multivariate analyses were performed.

Results: The frequency of *BCR::ABL1* was 9.01% [p = 0.097]. The *ETV6::RUNX1* gene rearrangement was observed in 37.0% *vs*. 52.6% (p = 0.168), and *KMT2A* gene rearrangement in 8.52% *vs*. 10.5% (p = 0.690) in $CD9^+$ and $CD9^-$ groups, respectively. The potential significance of *BCR::ABL1* suggests CD9's role in indicating the presence of this unfavourable genetic marker, while for *ETV6::RUNX1*, CD9 expression may be linked to a less positive genetic profile. Lymphadenopathy was significant in $CD9^+$ group, while bone marrow blast counts were notable in $CD9^-$ group. The survival rates did not significantly differ between the two groups.

Conclusion: CD9 can be used as a surrogate biomarker in predicting disease prognosis by recognising the patients with high-risk factors i.e., lymphadenopathy, elevated white blood cells, possible occurrence of *BCR::ABL1*, and the scarcity of *ETV6::RUNX1* within the $CD9^+$ group.

Key Words: *Immunophenotyping, Cytogenetics, Gene rearrangement, CD9 expression, Prognostic marker.*

How to cite this article: Meraj F, Mansoor N, Javed O, Jamal S. CD9 as a Potential Prognostic Biomarker in Paediatric B-Cell Acute Lymphoblastic Leukaemia. *J Coll Physicians Surg Pak* 2025; **35(02)**:191-196.

INTRODUCTION

Acute lymphoblastic leukaemia (ALL), the most common paediatric malignancy, accounts for more than 80% of acute leukaemias. B-cell lymphoblastic leukaemia (B-ALL) and T-cell lymphoblastic leukaemia (T-ALL) are the two main types of ALL, with the former accounting for around 85% of cases. Between the ages of 1 and 4 years, children have the highest incidence of ALL and then decline significantly during childhood (5–14 years).[1,2] Diagnostic and prognostic significance has been demonstrated for several B-ALL-related genetic aberrations. Translocations t (9;22), t (12;21), and translocations within the q23 region of chromosome 11 (t (11q23)), result in rearrangements of the genes, *BCR::ABL1*, *ETV6::RUNX1*, and *KMT2A* fusion genes, respectively.[3,4]

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Received: May 07, 2024; Revised: November 23, 2024; Accepted: December 28, 2024
DOI: https://doi.org/10.29271/jcpsp.2025.02.191

These chromosomal aberrations can result in leukaemic clones and hence lead towards leukaemia. The immunophenotypic expression on the blast cells can be used to differentiate abnormal leukaemic cells (blasts) through flow cytometry (FC).[5]

In B-ALL, detection of CD9 is considered as an aberrant expression.[6] The tetraspanin superfamily, which includes the 24–27-kDa cellular surface glycoprotein CD9, is an important tool for ALL diagnosis and minimal residual disease (MRD) monitoring.[7,8] Expression of CD9 fluctuates during the development of B-cell with upregulation in precursor B-cells, downregulation in mature B cells, and re-expression in plasma cells. Additionally, it influences cellular motility, transmits signals, facilitates apoptosis, and prevents metastasis.[9] Several solid tumours, including breast, colorectal and gastric cancer, as well as haematological malignancies, have been linked to the expression of CD9.[6,10,11]

Patients with CD9 positive ALL have higher neutrophil counts and advanced expression rates of the *BCR::ABL1* fusion gene.[6] A strong association of CD9 and an inverse relationship of CD9 with *ETV6::RUNX1*[7,12] and *KMT2A* rearrangement have also been reported.[12,13] The relationship between CD9 with clinical

characteristics and overall outcome in B-ALL patients has received little attention to date.

This study sought to assess CD9's potential as a biomarker for predicting prognostically significant *ETV6::RUNX1*, *BCR::ABL1*, and *KMT2A* fusion genes in B-ALL. Early recognition of these genetic markers can aid in tailoring more precise treatment strategies by early risk stratification leading to improved clinical outcomes. Additionally, in situations where cytogenetic testing is unavailable, immunophenotypic markers may serve as valuable tools for risk stratification and better therapeutic decision-making, ultimately improving clinical outcomes. The study aimed to evaluate the role of CD9 in predicting *ETV6::RUNX1*, *BCR::ABL1*, and *KMT2A* fusion genes with prognostic significance in B-ALL.

METHODOLOGY

A retrospective observational study of 488 B-ALL paediatric patients was carried out at the Indus Hospital from May 2020 to August 2022. All new consecutive cases of paediatric B-ALL diagnosed by flow cytometer were retrieved from the electronic medical records. The immunophenotyping and cytogenetics of all patients who meet the World Health Organisation (WHO) criteria for B-ALL were included in this study.[14] Children of both genders between 0 to 16 years, diagnosed with acute leukaemia of B-cell phenotype (CD19, CD79a positive) were included. Partially treated and adult cases were excluded. Ethical approval to report these cases was obtained from the Institutional Review Board of the Hospital [IHHN_IRB_2022_08_025, dated 29/08/2022].

Using the fluorochrome-conjugated antibodies, the expression of CD9 was observed by flow cytometry performed on BD FACS Canto II eight-colour flow cytometer and analysed by FACS DIVA software. CD9 was considered positive if 20 - 80% of the blasts were positive and less than 20% were negative. Fluorescent *in situ* hybridisation (FISH) was performed to determine the recurrent genetic abnormalities. It is a technique that allows the localisation of a specific DNA sequence on a chromosome. For the detection of *BCR::ABL1*, *ETV6::RUNX1*, and *KMT2A* fusion gene abnormalities, a multiprobe ALL panel was designed representing the chromosomal abnormalities t (9; 22) (q34; q11.2), t (12;21) (p13; q22), and 11q23, respectively.

All patients were treated with a modified Children's Oncology Group (COG) protocol. Event-free survival (EFS) was calculated from the start date of the treatment till the event occurrence, i.e. relapse of the disease. Relapse of the disease was defined as the return of disease after achieving haematological remission. The overall survival (OS), on the other hand, was calculated from the time of diagnosis to the time of death.

The data were entered and analysed using Microsoft Excel and the Statistical Package for the Social Sciences (SPSS) software version 23.0. For categorical variables, frequencies and percentages were calculated. Median and interquartile ranges (IQR) were calculated for numerical data which were found to be not normally distributed as per Kolmogorov–Smirnov test. Age was segregated into three categories according to their median age i.e., ≤3, >3 to ≤9, and >9 years.

Mann-Whitney U test was used to compare all continuous variables, while the Pearson's Chi-square test and Fisher's exact test were used to compare the two CD9 positive and negative groups to patient-related categorical variables. A p-value of <0.05 was considered statistically significant. For the estimation of the factors affecting OS and EFS, univariate and multivariate Cox-regression analyses were performed in CD9-positive patients.

RESULTS

The clinical and laboratory characteristics of 488 B-ALL patients according to $CD9^+$ (n = 469, 96%) and $CD9^-$ (n = 19, 3.9%) groups are summarised in Table I. The median age was 5 years (IQR 3-9.8) in $CD9^+$ while 6 years (IQR, 2.6-8) in $CD9^-$ group indicating that age did not influence the CD9 expression. The cumulative incidence of *BCR::ABL1* was 9.01% (n = 44), whereas the overall incidence of *BCR::ABL1* was 8.52% (n = 40) and 21.0% (n = 4) in $CD9^+$ and $CD9^-$ group, respectively (p = 0.097). The *ETV6::RUNX1* gene was observed in 33.9% (n = 159) and 52.6% (n = 10) patients in $CD9^+$ and $CD9^-$ groups, respectively (p = 0.168). The *KMT2A* gene rearrangement occurred in 8.52% (n = 40) in $CD9^+$ group and 10.5% (n = 2) in $CD9^-$ group (p = 0.690). Although the findings of this study did not reach statistical significance for *BCR::ABL1* (p = 0.097), they suggest that CD9 may play a role in indicating the presence of this unfavourable genetic alteration.

On the other hand, the p-value of *ETV6::RUNX1* was 0.168, suggesting that the absence of CD9 expression is more commonly noted in patients with *ETV6::RUNX1*. Interestingly, *KMT2A* appeared at a similar rate in both groups, indicating that CD9 expression may have no association (p = 0.690).

$CD9^+$ group exhibited a higher occurrence of lymphadenopathy compared to the $CD9^-$ group (p = 0.015*). No association of CD9 was seen on CD34 as its positive expression was noted in both $CD9^+$ (n = 326/ 69.5%) and $CD9^-$ (n = 14/73.6%) groups. The median bone marrow blast counts in the $CD9^-$ group were higher showing statistically significant difference between the two groups (p = 0.015*). No significant difference was observed in the rate of survival among $CD9^+$ and $CD9^-$ groups.

In CD9-positive patients, univariate analysis of EFS and OS revealed numerous important results. Hazard ratio (HR), which is a statistical measure used to compare the risk of an event occurring in one group relative to another group for EFS and OS, was calculated. The HR for event-free survival (EFS) was higher in patients under nine years, while for OS, the HR was higher in patients over three years of age. Additionally, patients between three and nine years had a higher HR in both EFS and OS. In univariate analysis, gender did not demonstrate a statistically significant impact on EFS but exhibited a significant effect on OS (HR: 1.921; 95% CI: 1.168-3.159, p = 0.010). However, multivariate analysis did not show this significance (p = 0.360).

Table I: Baseline characteristics among groups of CD9 expression.

Variables		CD9 (Positive)	CD9 (Negative)	p-value
Gender n (%)	Male	275 (58.6%)	13 (68.4%)	0.480[a]
	Female	194 (41.3%)	6 (31.5%)	
Age (years)	Median (IQR)	5 (3-9.8)	6 (2.6-8)	0.378[c]
BCR::ABL1 n (%)	Positive	40 (8.5%)	4 (21%)	0.097[a]
	Negative	402 (85.7%)	15 (78.9%)	
ETV6::RUNX1 n (%)	Positive	159 (37.0%)	10 (52.6%)	0.168[b]
	Negative	271 (63.0%)	9 (47.3%)	
KMT2A n (%)	Positive	40 (8.5%)	2 (10.5%)	0.690[a]
	Negative	402 (85.7%)	17 (89.4%)	
Fever	Yes	415 (88.4%)	17 (89.4%)	>0.999[a]
	No	54 (11.5%)	2 (10.5%)	
Lymphadenopathy	Yes	177 (37.7%)	2 (10.5%)	0.015[a]*
	No	292 (62.2%)	17 (89.4%)	
Bleeding	Yes	49 (10.4%)	2 (10.5%)	>0.999[a]
	No	420 (89.5%)	17 (89.4%)	
Bruises	Yes	48 (10.2%)	1 (5.2%)	0.709[a]
	No	421 (89.7%)	18 (94.7%)	
Abdominal pain	Yes	56 (11.9%)	3 (15.7%)	0.491[a]
	No	413 (88.0%)	16 (84.2%)	
Blood count parameters median (IQR)	BM Blasts (%)	51 (24-58)	66 (38-83)	0.015[c]
	WBC Count ($x10^9$/L)	20.4 (6.6-62.9)	9.9 (5.9-23.4)	0.086[c]
	Neutrophil (%)	5 (2-12)	4 (2-13.5)	0.685[c]
	Lymphocytes (%)	28 (11-58)	41.5 (30.7-58.5)	0.083[c]
	Haemoglobin (gm/dl)	7 (5.55-9)	5.6 (4.1-9.1)	0.240[c]
	Platelets ($x10^9$/L)	24 (13-50)	26 (15-95)	0.446[c]
MRD induction	Positive	113 (63.8%)	6 (54.5%)	0.535[a]
	Negative	64 (36.2%)	05 (45.5%)	
Outcome	Alive	406 (86.8%)	17 (89.4%)	>0.999[a]
	Expired	63 (13.4%)	2 (10.5%)	

**p-value <0.05 indicating significant association, a = Fisher's exact test, b = Pearson's Chi-Square, c = Mann-Whitney U test.*

Table II: Univariate and multivariate Cox-regression analyses of potential risk factors on EFS.

Variables		Cox-regression of EFS in CD9-positive patients			
		Univariate analysis		Multivariate analysis	
		HR [95% CI]	p-value	HR [95% CI]	p-value
Age (years)	≤3	1.245 [0.540, 2.870]	0.608	0.868 [0.268, 2.806]	0.813
	>3 to ≤9	1.321 [0.657, 2.657]	0.435	0.788 [0.277, 2.240]	0.655
	>9	0.619 [0.308, 1.246]	0.179	-	-
Gender (ref. female)	Male	1.053 [0.522, 2.122]	0.886	0.808 [0.297, 2.196]	0.676
WBC (ref. normal)	Abnormal	2.495 [0.858, 7.257]	0.093	2.435 [0.663, 8.937]	0.180
BCR::ABL1 (ref. negative)	Positive	5.391 [2.454, 11.842]	<0.001*	5.250 [1.788, 15.421]	0.003*
ETV6::RUNX1 (ref. negative)	Positive	0.398 [0.120, 1.321]	0.132	0.516 [0.067, 3.969]	0.525
KMT2A (ref. negative)	Positive	0.928 [0.124, 6.934]	0.942	-	-
MRD induction (ref. negative)	Positive	1.705 [0.626, 4.639]	0.296	1.086 [0.360, 3.275]	0.883

**Significant = <0.025 (Univariate), *Significant = <0.05 (Multivariate), HR = Hazard ratio, CI = Confidence interval.*

Table III: Univariate and multivariate Cox-regression analyses of potential risk factors on OS.

Variables		Cox-regression of OS in CD9-positive patients			
		Univariate analysis		Multivariate analysis	
		HR [95% CI]	p-value	HR [95% CI]	p-value
Age (years)	≤3	0.516 [0.313, 0.853]	0.010*	0.086 [0.007, 1.122]	0.061
	>3 to ≤9	1.358 [0.820, 2.250]	0.235	0.113 [0.008, 1.583]	0.105
	>9	1.507 [0.803, 2.827]	0.201	-	-
Gender (ref. female)	Male	1.921 [1.168, 3.159]	0.010*	1.769 [0.521, 6.007]	0. 360
WBC (ref. normal)	Abnormal	2.416 [1.093, 5.339]	0.029	2.005 [0.235, 17.115]	0.525
BCR::ABL1 (ref. negative)	Positive	0.524 [0.164, 1.673]	0.275	2.042 [0.412, 10.109]	0.382
ETV6::RUNX1 (ref. negative)	Positive	1.401 [0.821, 2.389]	0.216	-	-
KMT2A (ref. negative)	Positive	1.493 [0.640, 3.485]	0.354	32.313 [1.918, 544.257]	0.016
MRD induction (ref. negative)	Positive	36.506 [0.214, 6224.7]	0.170	146431.866 [0.000, 2.609E + 180]	0.954

**Significant = <0.025 (Univariate), *Significant = <0.05 (Multivariate), HR = Hazard ratio, CI = Confidence interval.*

The results found no significant correlation between EFS and aberrant white blood cell (WBC) count. Higher WBC counts for OS were relatively significant compared to EFS in the univariate analysis (HR: 2.416; 95% CI: 1.093-5. 339, p = 0.029) but did not maintain significance in multivariate analysis. Indicating a higher risk, *BCR::ABL1* positive status considerably influenced the EFS in both univariate (p = 0.001) and multivariate (p = 0.003) analyses. However, it did not have much effect on OS. Emphasising the need to monitor WBC counts and considering *BCR::ABL1* status in clinical decision-making for CD9-positive patients, the results of this study provide insightful analysis of the prognostic elements influencing patient outcomes. Univariate and multivariate Cox-regression analyses of potential risk factors on EFS and OS are shown in Table II, III, and Figure 1.

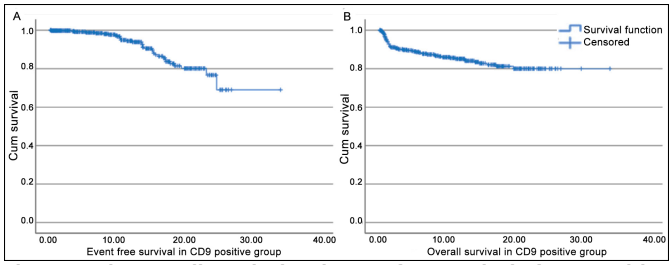

Figure 1: The overall survival and event-free survival of CD9-positive patients. (A) EFS curve of CD9-positive cases, indicating 70 to 80% of the patients who remained event-free during this period and (B) OS curve of CD9-positive patients showing 80% of cumulative survival.

DISCUSSION

In this study, the CD9$^+$ group exhibited a higher incidence of lymphadenopathy and elevated WBC counts. The potential association between the *BCR::ABL1* fusion gene and CD9 was identified with a value of 0.097, thus indicating a trend towards significance, suggesting a possible link that requires further investigation to confirm it. Conversely, the scarcity of *ETV6::RUNX1* within the CD9$^+$ group suggests a less favourable outlook for these patients. Nevertheless, further exploration of these findings in a larger cohort of negative cases is warranted to establish their clinical significance. Previous research has found that the clinical and prognostic significance of CD9 expression varies depending on the kind of tumour.[15]

The median age of patients was 5.0 (3-9.8) years. These findings are in accordance with an earlier study.[16] There was a male preponderance in the present study, which Blunck *et al*, Touzet *et al*, and Wang *et al*. also found in their studies.[7,17,18] Koh *et al*. also found that male gender was associated with increased CD9 expression (OR = 0.76, 95% CI 0.59-0.99, p = 0.044).[15]

Among 488 patients, a significant association of CD9 with *BCR::ABL1*, *ETV6::RUNX1*, *KMT2A* was not found but *BCR::ABL1* was seen more frequently in the CD9$^+$ group followed by *KMT2A* compared to *ETV6::RUNX1*. Similarly, a study conducted in China shows *BCR::ABL1* as more frequent in the CD9$^+$ group.[6] According to Tsagarakis *et al*, there is a direct correlation between CD9 expression with *BCR::ABL1* and *KMT2A*, similar findings of CD9 expression with *BCR::ABL1* were also confirmed by others.[4,13] The overall incidence of *ETV6::RUNX1* reported in the present study was 33.9%, compared to several other studies from India as well as from Western world presenting similar incidence of *ETV6::RUNX1*.[19] A study conducted by Blunck *et al*. reported 23.6% cases of *ETV6::RUNX1*.[7] Other reports documented a low occurrence of CD9 positivity in patients with *ETV6::RUNX1*.[2,3,15,20]

In this study, lymphadenopathy was the only significant clinical finding observed in CD9$^+$ compared to CD9$^-$ group, while the difference between the two groups for all other clinical findings was insignificant. Most of the studies also showed similar findings.[6,21,22] The current study revealed equal distribution of CD34 expression in both groups, while a Chinese study found a higher percentage of CD34 expression in B-ALL cases with CD9 expression.[6]

CBC showed lower haemoglobin and platelets whereas WBC counts were significantly higher. These findings are in concordance with a study conducted in India.[19] It was observed that WBC counts had no significant impact on EFS while a comparatively better significance was observed on OS. A study conducted in China, reported that WBC counts (HR = 1.432, 95% CI: 0.783-2.620) showed an increased risk of mortality[6] while others have yielded insignificant results.[19] The analysis showed that the HR for EFS was higher in patients under nine years, while for OS, the HR was higher in patients over three years of age. Additionally, patients between three and nine years have a higher HR in both EFS and OS. The multivariate analysis in this study revealed that the findings were in concordance with the results reported by Leung *et al*.[20] Another recent study published by Leung *et al*. clearly indicates the inferior survival of patients with CD9 expression and suggested to incorporate the targeted therapy for CD9 blockade.[23]

This study only reflects the findings of the paediatric population since the centre / hospital does not provide adult oncological services. However, most of the observations are consistent with other studies that have looked at a larger population. Another limitation is the small sample size of CD9$^-$ cases. Further studies incorporating larger cohorts can be more reflective of clinical significance.

CONCLUSION

This research highlighted the significance of CD9 expression in B-ALL. It emerges as a valuable tool for recognising patients at high risk, especially those with lymphadenopathy, elevated WBC counts, and possible occurrence of *BCR::ABL1*. Conversely, the scarcity of *ETV6::RUNX1* within the CD9$^+$ group suggests a less favourable outlook for these patients. These findings offer insights that could assist in risk assessment and tailored treatment strategies for B-ALL patients.

ETHICAL APPROVAL:

The ethical approval was acquired prior to the investigation from the Institutional Review Board of the Indus Hospital and Health Network, Karachi, Pakistan (IHHN_IRB_2022_08_025).

PATIENTS' CONSENT:

The study does not involve direct interaction with patients and thus acquiring consent was not applicable.

COMPETING INTEREST:
The authors declared no conflict of interest.

AUTHORS' CONTRIBUTION:
FM: Conceived, designed, analysed, drafted the manuscript, and supervised data collection.
NM: Critical review and editing of the manuscript.
OJ: Participated in reviewing the possible revisions.
SJ: Critical review and final approval.
All authors approved the final version of the manuscript to be published.

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