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Original Article

MEF2C expression, but not absence of bi-allelic deletion of TCR gamma chains (ABD), is a predictor of patient outcome in Indian T-acute lymphoblastic leukemia

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Abstract: Emerging evidence suggests existence of three prognostically relevant molecular entities among immature T-ALL-early thymic precursor ALL (ETP-ALL), T-ALL with the absence of biallelic deletion of TCRγ chains (ABD) and *MEF2C* (Myocyte Enhancer Factor 2C) high T-ALL. However, the usefulness of ETP-ALL immunophenotype and assessment of ABD for this purpose has been questioned and, *MEF2C* has not been studied in much detail. In this prospective analysis of 143 T-ALL patients, we evaluated the mutual association of these three entities and also determined how immunophenotypically-defined poor prognosis immature T-ALL relates to these entities. We found that all three of them, especially ABD, nearly completely characterized the immature group. High *MEF2C* expression reflected ETP-ALL somewhat poorly and a few ABD and *MEF2C*-high patients had non-immature immunophenotype-findings, that though in accord with published literature, call for exploration per T-cell receptor (TCR) classification scheme. ETP-ALL and *MEF2C* high but not ABD had a higher frequency of minimal residual disease positivity and poor event-free survival. *MEF2C* high, not ETP-ALL immunophenotype or ABD, had poorer overall survival. The value of ETP-ALL immunophenotype and *MEF2C* status, as indicators of poor treatment response, needs further evaluation for possible incorporation in standard T-ALL management practice.

Keywords: T-ALL, *MEF2C*, ABD, ETP-ALL

Introduction

T-acute lymphoblastic leukemia (T-ALL) is an aggressive, molecularly heterogeneous malignancy caused by the leukemic transformation of T-cell progenitors at different stages of maturation, that can be recognized immunologically and as well as by gene expression profiling (GEP) [1, 2]. Immunologically-defined immature T-ALL (sCD3-, CD1a-) has been known to be a poor prognostic group requiring a more intensive therapeutic regimen [3-5]. Gene expression studies have identified immature or early immature T-ALL to be a transcriptionally distinct entity that arrests at a very early stage of development, overexpresses *LYL1,* and has gene expression signature most related to hematopoietic stem cells and myeloid progenitors [2, 6, 7]. Several reports suggest that immature T-ALL is also heterogenous at the molecular level and three subgroups of immature T-ALL have been widely recognized-early thymic precursor ALL (ETP-ALL) [8], T-ALL with the absence of bi-allelic deletion (ABD) at T-cell receptor gamma (*TCRγ)* locus [9] and *MEF2C* (Myocyte Enhancer Factor 2C)-driven T-ALL [10, 11]. Early thymic precursor ALL (ETP-ALL) was described as an entity, whose immaturity was reflected in its sharing a GEP with normal early thymic precursors [8]. Another subgroup was reported whose immaturity was evidenced by the fact that, like most normal human prothymocytes, it did not rearrange the *TCRγ* gene, and hence had ABD at TCRγ locus [9]. The third subgroup was shown to be driven by the overexpression of transcription factor *MEF2C* and is considered

the same as ETP-ALL on account of their shared GEP [10, 11].

Recognition of high-risk T-ALL groups by tests that, unlike GEP, are easily doable, is important from the standpoint of prognostication and risk-adaptation of therapy; all three entities lend themselves to such testing. However, the promise and value of easy recognition of ETP-ALL by its characteristic immunophenotype (CD5 dim/negative, CD1a negative, CD8 negative and expression of one or more myeloid and stem cell markers) has been diminished by, among other reasons, the demonstration that immunophenotype significantly underestimates ETP-ALL uncovered by GEP [6, 8, 11, 12], pointing to a need for additional testing. Similarly, there is a lack of agreement about the value of testing for ABD by Q-PCR, which has been recommended by clinical trials for having the potential to predict induction failure [9, 13, 14]. *MEF2C* has not been evaluated in this respect [12]. Though they all define immaturity in T-ALL, correspondence between them, paradoxically, is not perfect [9, 11, 12]. The aim of this study was, therefore, to examine the clinical relevance of determination of *MEF2C* gene expression, ETP immunophenotype and ABD, in T-ALL patients.

Materials and methods

Patient samples

Diagnostic peripheral blood (PB)/bone marrow (BM) samples were collected from newly diagnosed 143 T-ALL patients before the start of initial therapy. Informed consent was given by patients or parents/guardians to use the left-over diagnostic material for research purposes as approved by the institutional ethics committee.

Immunophenotyping

Immunophenotyping was done in all patients (n = 143) on BM/PB samples collected in EDTA using the stain-lyse-wash method. The antibodies used were those emphasized for a diagnosis of ETP-ALL: CD7, CD3 (both surface and intracytoplasmic), CD2, CD45, CD5, CD8, CD1a, CD13, CD33, CD117, HLA-DR, CD34, CD65 and CD11b. The diagnosis of ETP-ALL was based on immunophenotypic criteria: CD5 dim/negative, CD1a negative, CD8 negative and expression of one or more myeloid and stem cell markers [8].

Assessment of absence of bi-allelic deletion of TCR gamma

DNA was isolated from PB/BM samples by using a DNA extraction kit from Thermo Fisher Scientific (Massachusetts, USA). The status of *TCRγ* deletion was determined using the protocol designed by Gutierrez et al., 2010 [9]. The quantitative DNA-PCR primer sequences were *TCRG-VJ* forward, CATCCTCACTTTCCTGCTTCTTC, and *TCRG-VJ* reverse, CCAAGGTGAATCCCTACATGCT; control Q-PCR primers were in the Anillin Actin Binding Protein (*ANLN)* locus, located approximately 1.9 Mbp downstream of *TCRγ* at 7p15-14; *ANLN* forward, 5'-AAATTCTGCCCTTTGCTTGTTT-3' and *ANLN* reverse, 5'-GAAAGCAACCACAGAGAATATGTAAGTAA-3'. All PCR reactions were performed in triplicate. Results of the *TCRγ* quantitative PCRs were reported as fold-change compared to the *ANLN* Q-PCR. Patients were assigned to the ABD group if the *TCRγ*: *ANLN* fold change was >0.5 and diagnostic blast count >85% (to exclude the lack of deletion due to contamination by non-leukemic cells), non-ABD if <0.35 and indeterminate if between these values [9].

Determination of MEF2C expression

RNA was extracted using TRIzol (Thermo Fisher Scientific, Massachusetts, USA) reagent using the manufacturer's instructions. *MEF2C* gene expression was quantified using the following primers and SYBR Green dye: *MEF2C*-Forward: 5'-GCGCTGATCATCTTCAAC-3' and *MEF2C*-Reverse: 5'-CTTTGCCTGCTGATCATT-3'. The housekeeping genes used were: *β-actin*, *ABL1,* and *GPI*. In all cases, samples were run in triplicates. Ct values were normalized with housekeeping genes. For *MEF2C* gene expression, the T-ALL cases were dichotomized at its median with patients classified as low gene expression if they had expression values within the lower 50% and as high gene expression if they had gene expression values within the upper 50%.

Determination of minimal residual disease

Minimal residual disease (MRD) was assessed in the post-induction BM samples by bulk lysis method using the antibody panel: CD8FITC

(Fluorescein isothiocyanate), CD7PE (Phycoerythrin), cytoplasmic CD3ECD (PE-Texas Red), CD34PC5.5 (PE-Cyanin 5.5), CD5PC7 (PECyanin 7), CD4APC (Allophycocyanin), CD45AF700 (Alexa Fluor 700), CD38AF750 (Alexa Fluor 750) (Beckman Coulter [BC], Hialeah, FL, USA); CD16/CD56 BV510 (Brilliant Violet 510) and surface CD3 BV421 (Brilliant Violet 421) (Biolegend, San Diego, CA). For all specimens, ten-color FCM was performed on a Coulter Gallios instrument (Beckman Coulter [BC], Hialeah, FL, USA). At least 1 million events were acquired in each case. Patients with MRD >0.01% were classified as MRD positive; <0.01% as MRD negative and inevaluable if the BM sample was diluted and provided PB only.

Treatment

In this study, 84 patients were treated with ICICLE protocol (CTRI/2015/12/006434), 28 with Berlin-Frankfurt-Muenster-90 (BFM-90) protocol, and 3 with the hyper-CVAD (cyclophosphamide, vincristine, adriamycin, and dexamethasone) protocol. Twenty-eight patients did not take any treatment. Two patients died during induction chemotherapy.

Statistical analysis

Fisher's exact test for categorical data and non-parametric Mann Whitney test for continuous variables were used to compare baseline clinical variables across groups. A *P* value <0.05 (two-sided) was considered significant.

Response to prednisolone was assessed by the examination of peripheral blood at day 8 of initiation of prednisolone therapy. The patients were divided into prednisolone-sensitive and-resistant based on the presence of <1000/μL and ≥1000/μL blasts, respectively.

The median follow-up was 20 months. After excluding 28 patients who did not receive treatment, 115 patients were included for the analysis of event-free survival and overall survival.

Event-free survival (EFS) was defined as the time from diagnosis to the date of the last follow-up in complete remission or the first event (i.e., induction failure, relapse, secondary neoplasm, or death from any cause). Failure to achieve remission due to non-response was considered an event at time zero. Overall survival (OS) was defined as the time from diagnosis to death or the last follow-up. The Kaplan-Meier method was used to estimate survival rates, with the differences compared using a two-sided log-rank test. Cox proportional hazard models were constructed for EFS and OS and used for univariate and multivariate analyses. Covariates included in the full model of OS and EFS were sex, white blood cell count (WBC) (≤50×10^9/L, ≥50×10^9/L), and age (<10 years vs. ≥10 years), *MEF2C* gene expression, immunophenotype, central nervous system (CNS) involvement, response to prednisolone treatment, BM remission status and presence of MRD after the end of induction chemotherapy. All analyses were performed using the SPSS statistical software package, version 20.0/STATA software, version 11.

Results

Of the 143 patients studied, the material with sufficient quality and quantity was available for determination of ABD in 115, *MEF2C* in 105, and immunophenotyping (including determination of ETP-ALL status) in all 143 cases, with 89 cases studied for assessment of all these parameters. Patient samples for MRD were available in 95 cases.

There were 105 children (<17 years old) and 38 adults (>17 years old) patients. There were 127 males and 16 females. The mean hemoglobin, WBC and platelet count was 9.2 g/dl, 121.18×10^9/L, and 89.1×10^9/L, respectively. Per European Group for the Immunological Classification of Leukemias (EGIL) criteria [1], there were 67 (46.8%) immature (pro-T-ALL 27; pre-T-ALL 40), 56 (39.2%) cortical and 20 (14.0%) mature T-ALL cases. Also, ETP-ALL immunophenotype was identified in 19/143 cases (13.3%; a subset of 10/27 pro-T-ALL and 9/40 pre-T-ALL) and $CD5^+CD1a^-CD8^-$ pre-T-ALL immunophenotype in 31/143 cases (21.6%; a subset of 40 pre-T-ALL). Concerning ABD status, patients were classified as ABD 42/115 (36.5%), non-ABD 54/115 (47.0%) and indeterminate 19/115 (16.5%). MRD was positive in 27/95 (28.4%) cases, negative in 59/95 (62.1%) cases, and not evaluable (because of the diluted sample) in 9 (9.5%) cases.

Table 1. ABD status and *MEF2C* expression in T-ALL

Parameters	Non-ABD N = 54 (%)	ABD N = 42 (%)	*P* value	*MEF2C* low N = 52 (%)	*MEF2C* high N = 53 (%)	*P* value
Age (in years)						
<10	16 (29.63)	13 (30.95)	0.889	17 (32.7)	16 (30.2)	0.924
≥10	38 (70.37)	29 (69.05)		35 (67.3)	37 (69.8)	
Sex						
Male	51 (94.44)	37 (88.1)	0.26	46 (88.5)	46 (86.8)	0.727
Female	3 (5.56)	5 (11.90)		6 (11.5)	7 (13.2)	
WBC count ($\times 10^9$/L)						
<50	23 (42.59)	30 (71.43)	0.005	26 (50)	33 (62.3)	0.337
≥50	31 (57.41)	12 (28.57)		26 (50)	20 (37.7)	
Mediastinal mass						
No	24 (70.6)	34 (70.8)	1.00	31 (70.5)	33 (71.7)	0.96
Yes	10 (29.4)	14 (29.2)		13 (29.5)	13 (28.3)	
CNS disease at diagnosis						
No	50 (92.59)	41 (97.62)	0.379	45 (95.7)	41 (93.2)	0.34
Yes	4 (7.4)	1 (2.38)		2 (4.3)	3 (6.8)	
Immunophenotypic subtype of T-ALL						
Immature	9 (16.67)	34 (80.95)	<0.001	16 (30.8)	32 (60.4)	0.001
Cortical	34 (62.96)	6 (14.28)		30 (57.7)	17 (32.1)	
Mature	11 (20.37)	2 (4.76)		6 (11.5)	4 (7.5)	
ETP immunophenotype						
Yes	5 (9.3)	8 (19%)	0.23	3 (5.8)	13 (24.5)	0.009
No	49 (90.7)	34 (81%)		49 (94.2)	40 (75.5)	
$CD5^+$ Pre-T immunophenotype						
Yes	2 (3.7)	17 (40.5)	<0.001	9 (17.3)	10 (18.9)	0.22
No	52 (96.3)	25 (59.5)		43 (82.7)	43 (81.1)	
Prednisolone response						
Sensitive	25 (73.5)	22 (81.5)	0.549	25 (73.5)	22 (62.9)	0.019
Resistant	9 (26.5)	5 (18.5)		9 (26.5)	13 (37.1)	
Bone marrow remission status after induction therapy						
Yes	45 (83.33)	29 (69.05)	0.182	42 (80.8)	36 (67.9)	0.222
No	3 (5.56)	3 (7.14)		1 (1.92)	7 (13.21)	

*Association between ABD status and patient characteristics (**Table 1**)*

34/42 (81.0%) ABD positive patients had immature immunophenotype; the remaining 8 (19.0%) had non-immature immunophenotype-6 cortical and 2 mature. For 29 patients, in which both ABD and MEF2C status were assessed, majority 18/29 (62.1%) ABD positive patients were classified as *MEF2C* high, the latter were distributed equally (18/36; 50.0%) between ABD positive and negative cases. Whereas 8/42 (19.0%) ABD had ETP-ALL, 8/13 (61.5%) ETP-ALL had ABD. 34/43 (79.07%) patients with immature immunophenotype and 17/19 (89.5%) $CD5^+$ pre-T-ALL patients had ABD. Of the 9 ABD negative immature T-ALL patients 6 (66.7%) had *MEF2C* high and/or ETP immunophenotype, leaving only 3/9 (33.3%) without evidence of the three immature T-ALL entities. Overall, there were only 3/43 (6.9%) immature T-ALL patients who did not show features of ABD, ETP-ALL immunophenotype, or *MEF2C* high.

WBC count at diagnosis was lower in ABD group as compared to non-ABD group ($P = 0.005$). There was no statistically significant associa-

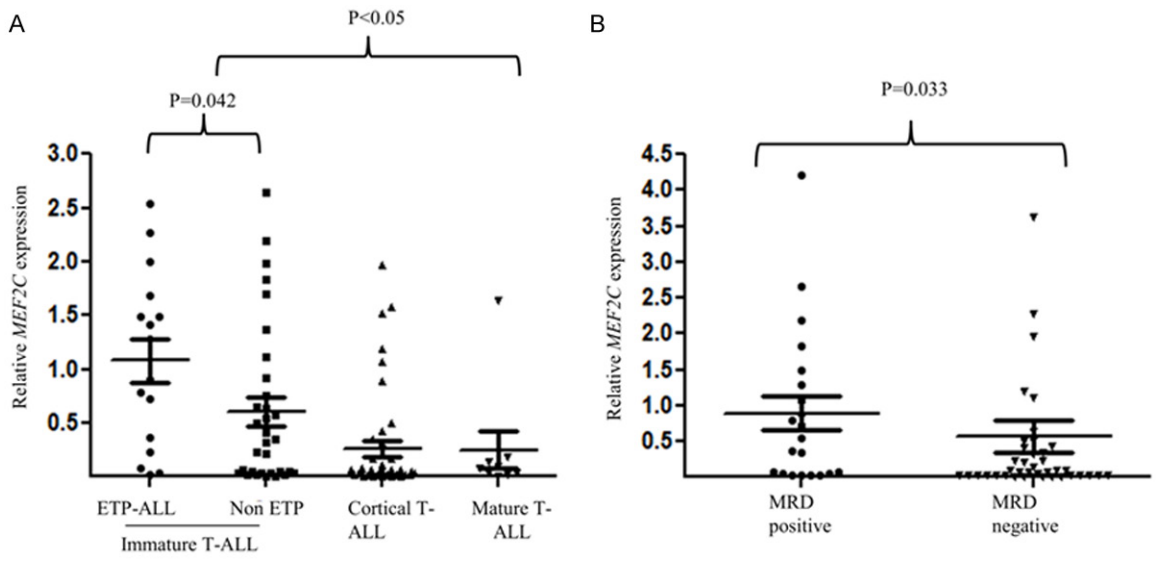

Figure 1. Plot demonstrating expression of *MEF2C* gene in relation to (A) various immunophenotypic subtypes of T-ALL, (B) MRD positivity.

tion between ABD status with sex (P = 0.26), age (P = 0.889), prednisolone response (P = 0.549), CNS disease (P = 0.379), post-induction BM remission status (P = 0.182) and MRD status (P = 0.607).

*Association between MEF2C expression and patient characteristics (**Table 1**)*

Patients with high *MEF2C* expression more frequently had immature immunophenotype as compared to patients with low expression (60.4% vs. 30.8%; P = 0.001). While 32/53 (60.4%) *MEF2C* high patients were immature, the remaining 21/53 (39.6%) *MEF2C* high patients had non-mature immunophenotype (cortical 17/53; 32.1% and mature 4/53; 7.5%). However, 14/17 cortical and all 4 mature T-ALL cases had blast percentage <85%. Corrected for contamination with maturing myeloid cells, therefore, high *MEF2C* was present only in 3/17 (17.6%) patients with cortical immunophenotype and none with mature immunophenotype.

Of the immature T-ALL patients, 32/48 (66.7%) had *MEF2C* high. *MEF2C* gene expression was higher in immature T-ALL patients who had ETP-ALL immunophenotype as compared to patients without this immunophenotype (P = 0.042). Among ETP-ALL patients 13/16 (81.2%) were *MEF2C* high (P = 0.009) and 13/53 (24.5%) *MEF2C* high had the ETP-ALL immunophenotype. **Figure 1A** shows the relative expression of the *MEF2C* gene with immunophenotypic subtypes of T-ALL.

Prednisolone resistance was more common in patients with high *MEF2C* expression (54.2% versus 37.5%; P = 0.019). There were no significant differences between patients with low and high *MEF2C* expression with respect to pre-treatment WBC (P = 0.337), age (P = 0.924), sex (P = 0.727), CNS involvement (P = 0.34), $CD5^+CD8^-CD1a^-$ pre-T-ALL (P = 0.22) and BM remission status (P = 0.22). Median level of *MEF2C* expression in MRD positive patients was higher as compared to MRD negative patients (0.5 versus 0.07; P = 0.033) (**Figure 1B**).

Association of immunophenotype with treatment response

There was no association between prednisolone response and immature, cortical, and mature T-ALL cases (P = 0.114) and $CD5^+CD8^-CD1a^-$ pre-T-ALL immunophenotype (P = 1.000). Although not statistically significant (P = 0.062), ETP-ALL immunophenotype (50%) was found to be better associated with prednisolone resistance as compared to non-ETP ALL cases (21.6%). BM remission rate was higher in non ETP-ALL patients (78.2%) as compared to ETP-ALL cases (57.9%) (P = 0.002). There was no association of $CD5^+CD8^-CD1a^-$ pre-T-ALL immunophenotype and remission rate (P = 0.612).

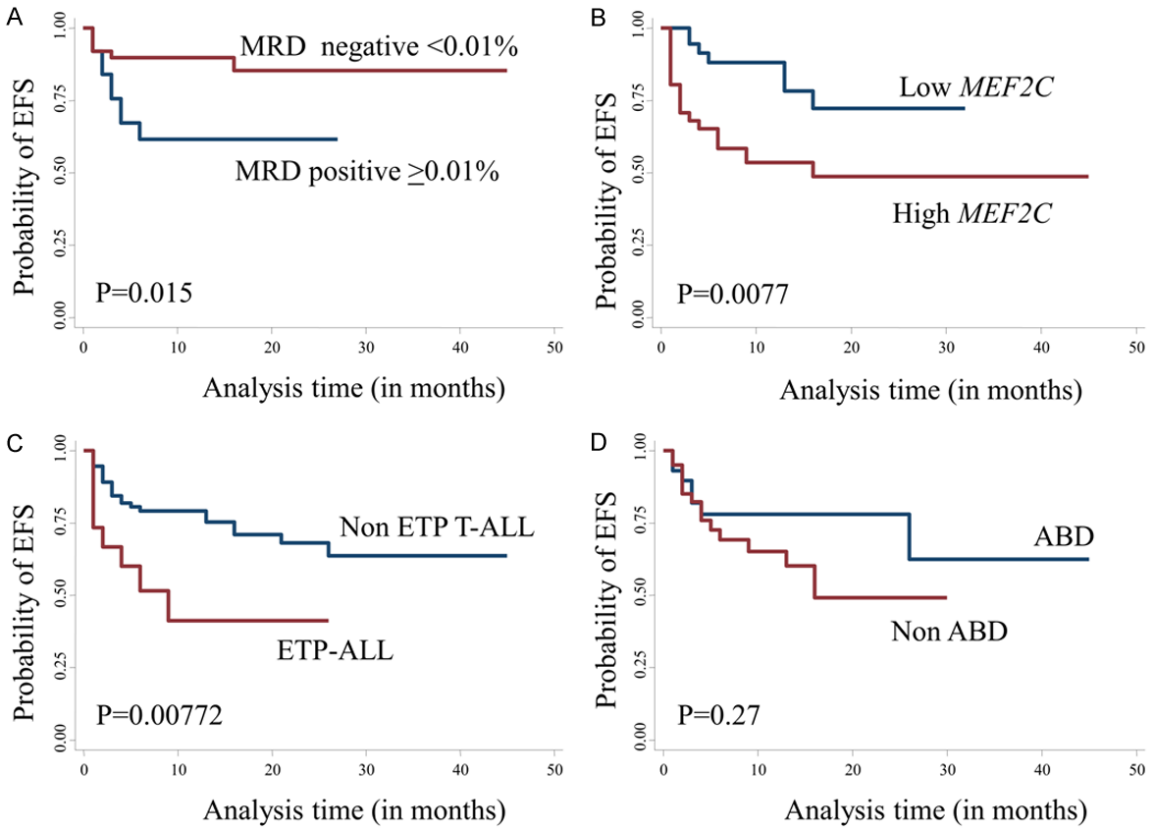

Figure 2. Kaplan Meier curves for event free survival analysis for T-ALL patients grouped according to (A) MRD status, (B) *MEF2C* expression, (C) ETP-ALL immunophenotype and (D) ABD status.

ETP-ALL immunophenotype patients (61.5%) more likely had MRD positivity in the post-induction BM as compared to non-ETP-ALL patients (26%) (P = 0.011). $CD5^{+}CD8^{-}CD1a^{-}$pre-T-ALL did not exhibit any association with MRD positivity (P = 0.120).

Survival analysis

Out of 143 patients included in the study, 28 (19.58%) patients discontinued without taking treatment. Survival analysis for EFS and OS was done in the remaining 115 patients (**Figures 2** and **3**).

On univariate analysis, National Cancer Institute (NCI) high risk, ETP-ALL immunophenotype, MRD positivity, and *MEF2C* high expression were associated with poor EFS (P = 0.041, 0.0072, 0.015 and 0.007, respectively) (**Table 2**). In multivariate analysis, ETP-ALL was a borderline significant predictor of EFS (P = 0.08) (**Table 3**). For overall survival, only the high *MEF2C* expression was a significant predictor of poor outcome (P = 0.012).

Discussion

T-ALL leukemic progenitors are a heterogeneous lot, distinguished by their immunophenotype [1] as well as molecular characteristics [2, 6-10]. Immature T-ALL has long been of interest owing to the poor prognosis it confers and the more intensive therapeutic regimen it needs [3-5]. Recognizing the three entities that come under the rubric of immature T-ALL by easily doable laboratory tests will help in better stratification of patients for clinical studies. In this regard, the value of ETP-ALL testing by immunophenotyping and ABD by RQ-PCR needs more clarity [6, 11-14], while *MEF2C* has not been studied in detail. Also, how these entities correspond with immunophenotypically defined T-ALL groups, has not been comprehensively studied, which served as aim for the current study [1].

Association between immature, ABD, ETP, and MEF2C: Approximately 80% of our immunophenotypically defined immature T-ALL patients

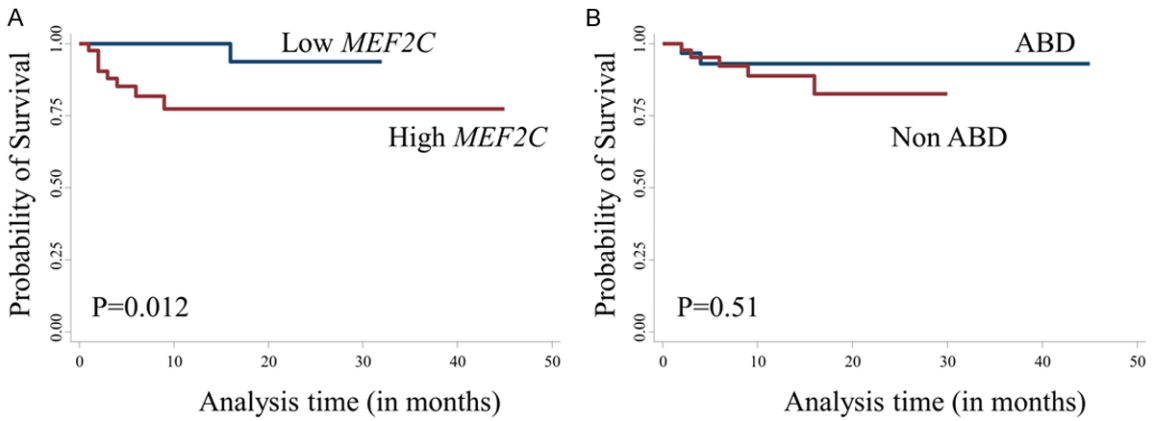

Figure 3. Kaplan Meier curves for overall survival analysis for T-ALL patients grouped according to (A) *MEF2C* expression and (B) ABD status. The overall survival analysis was done in 115 patients as 28 patients did not take treatment.

Table 2. Univariate analysis for event free survival and overall survival according to immunophenotype, ABD status, *MEF2C* gene expression and selected variables in T-ALL patients at AIIMS.

Variables	No. of Patients	Event free survival				Overall survival			
		EFS (%)	Hazard ratio	95% CI	*P* value	OS (%)	Hazard ratio	95% CI	*P* value
Age									
<10 years	37	69.57	0.67	0.30-1.51	0.33	93.69	0.607	0.13-2.92	0.52
≥10 years	78	56.44	1			88.71	1		
Sex									
Male	103	57.90	1			90.34	1		
Female	12	72.92	0.77	0.23-2.52	0.65	90	0.997	0.12-7.98	0.99
WBC count (×10^9/L)									
<50	71	62.89	1			89.98	1		
≥50	44	54.83	1.51	0.75-3.02	0.23	90.59	1.23	0.33-4.61	0.75
NCI risk group									
Standard	22	83.33	1			100	1	-	-
High	93	55.19	3.87	0.92-16.21	0.041	88.17			0.14
Mediastinal mass									
Yes	33	64.66	1.29	0.61-2.74	0.48	90.51	1.23	0.30-4.94	0.76
No	82	59.13	1			89.16	1		
ETP immunophenotype									
Yes	16	41.14	2.84	1.26-6.42	0.0072	83.33	1.88	0.39-9.11	0.42
No	99	63.64	1			91.36	1		
CD5$^+$ Pre T-ALL immunophenotype									
Yes	23	62.39	0.789	0.32-1.93	0.59	94.74	0.47	0.058-3.78	0.46
No	92	59.10	1			88.84	1		
MRD n=95 (evaluable in 85 cases) these 10 patients were excluded for statistical analysis									
Positive	26	61.60	1			91.48	1		
Negative	56	85.32	0.304	0.11-0.86	0.015	94.29	0.42	0.058-3.01	0.37
ABD (n=75)									
Yes	31	62.37	1			93.05	1		
No	44	49.17	1.63	0.66-4.00	0.27	82.54	1.70	0.33-8.81	0.51
MEF2C (n=83)									
Low	41	72.23	1			93.75	1		
High	18	48.68	3.02	1.26-7.28	0.0077	77.27	8.96	1.11-71.99	0.012

Table 3. Multivariate analysis for EFS

Variable	Hazard ratio	95% CI	*P* value
NCI risk, standard vs. high	3.43	0.44-26.86	0.24
ETP-ALL vs. non ETP-ALL	3.105	0.85-11.29	0.08
MRD, positive vs. negative	0.65	0.18-2.38	0.512
MEF2C expression, low vs. high	2.22	0.57-8.55	0.248

CI: Confidence of interval.

and, within this group, approximately 90% of $CD5^+$ pre-T-ALL, had ABD; *MEF2C* high present in 2/3 of immature T-ALL was thus largely co-expressed with ABD.

In the roughly 1/5 immature T-ALL patients who were ABD negative, 2/3 had *MEF2C* high and/or ETP immunophenotype. Thus, only 1/3 of the latter and overall, some 7% of the entire immature group, was without evidence of the three immature T-ALL entities. The three immature T-ALL entities therefore, fairly completely characterized the poor prognosis immunophenotypic immature group. Overall, these findings also show ABD to be a very good molecular correlate of immature T-ALL. Taken together with the fact that the usefulness of $CD5^{-/weak}$ as originally defined [8] has been questioned [9, 11], excellent correspondence of $CD5^+$ pre-TALL with ABD, supports the delineation of early immature T-ALL on parameters broader, hence $CD5^{+/-}$, than those suggested by the strict immunophenotypic criteria of ETP-ALL alone. $CD5^{+/-}$ rather than $CD5^{-/weak}$, is indeed how early immature T-ALL was defined in a study that showed its transcriptomic likeness with myeloid tumors [15].

Some observations-association of ABD and *MEF2C* high, both markers of immaturity, with non-immature groups-cortical and mature, imprecise correspondence between *MEF2C* and ABD, and *MEF2C* high not limited to ETP-ALL. Of the 8 (approx. 1/5) ABD patients who had non-immature immunophenotype, 6 were cortical and 2 mature. The original report on ABD [9] does not expressly comment on the correlation of ABD with immunophenotype; perusal of their data, however, shows a good proportion of their ABD patients to be having cortical or mature immunophenotype, including one with both CD1a and sCD3. Thus ABD, despite its conceptual underpinnings, is immunophenotypically not always immature; what appears as a discrepancy in our observations can, therefore, be explained by this.

MEF2C likewise, figured in our cortical T-ALL subgroup. This kind of discrepant finding has been noted in one patient in the first report on the entity of ETP-ALL [8] and has been explained also by possible contamination of the patients' BM samples with myeloid precursors [13]. Corrected for contamination with maturing myeloid cells (blasts <85%), we found high *MEF2C* in a small proportion of non-immature T-ALL-just 3/17 (17.6%) patients with cortical immunophenotype. A study evaluating the relationship among the three immature T-ALL entities found that only 40% of immature cluster *MEF2C* patients had not rearranged TRG@ loci (hence were ABD positive), implying that the remaining 60% immature cluster patients had rearranged their TRG@ (hence ABD negative) [11]. Once again, though there is no comment specifically in this report about whether the immature cluster *MEF2C* patients who had rearranged TRG@ (ABD negative), displayed CD1a cortical immunophenotype, given temporal changes in the developing human thymocyte, such a possibility cannot be ruled out. As pointed out by a previous study [9], though rare developing human $CD34^+$, $CD38^+$, $CD1a^-$ prothymocytes are known to rearrange TCRγ, this becomes common only at the $CD34^+$, $CD38^+$, $CD1a^+$ prethymocyte stage [16, 17].

Overall, what appears as discrepant or unexpected findings, therefore, may be a reflection of the heterogeneity of T-ALL as was reported in an Eastern Cooperative Oncology Group study in which 9 of 53 T-ALL patients manifested overlapping patterns of cell surface antigen expression [18]. Colomer-Lahiguer et al., 2017 [12], made similar observations concerning *MEF2C* [12]. In this connection, it may be fruitful in future studies to evaluate T-ALL patients employing the TCR classification scheme that, in contrast to the EGIL classification, defines in a different manner and more detail, the developmental stages of T-cells analogous to specific stages in normal T-lymphopoiesis [19]. It has been demonstrated that though both schemes distinguish immature, intermediate, and mature thymic stages of development, only about 50% of the T-ALL patients can be assigned

to equivalent development stages using the two systems [20, 21].

Most, approx. 2/3, ABD patients had *MEF-2C* high while *MEF2C* was distributed equally (50%), between ABD positive and negative cases, suggesting that these two molecular markers of immaturity do not exhibit precise correspondence. Similar observations were reported by Gutierrez et al., 2010 [9], who found that while nearly all ABD patients had the ETP-ALL gene expression signature, 50% of patients with ETP-ALL, by expression profiling, did not have ABD.

In our study ETP-ALL was defined by immunophenotype per the original recommendations [8]; status of CD5 as a defining feature of true ETP-ALL, that is, ETP-ALL recognized by GEP, however, has been questioned [9-11]. Our observation that only approx. 25% of our *MEF2C* high patients had ETP-ALL, signifies that ETP-ALL under-represents *MEF2C* high patients, which is in line with observations that ETP-ALL by immunophenotype, especially concerning CD5, strongly underestimates ETP-ALL recognized by gene signature [9-11].

Clinical correlates: We found an association between WBC count and ABD status (P = 0.005). The T-ALL patients with ABD had lower WBC as compared to T-ALL patients with a homozygous deletion of the TCR-γ gene. These findings were akin to those found by Farah et al., 2018 [13].

The value of ABD as a determinant of treatment outcome [9, 14, 22] has been questioned in a recent Medical Research Council UK Acute Lymphoblastic Leukemia 2003 study of pediatric/adolescent T-ALL, which found that ABD status did not add further prognostic information nor did it justify treatment escalation beyond what had already been inferred by MRD analysis using a risk-adapted protocol [13]. Similar to this, we did not find any association between ABD status and post-induction MRD, EFS, and OS.

MEF2C is a gene encoding for myocyte enhancer factor 2C, a member of the MADS-box transcription factor family that includes the MEF2A-D genes, which help in the regulation of skeletal muscle development [23]. *MEF2C* is highly expressed in hematopoietic stem cells and common myeloid progenitors or common lymphoid precursors. During normal intra-thymic T-cell development *MEF2C* is expressed at pre-DN1 and DN1 subsets and its expression drops significantly beyond DN2 stage7, suggesting the central role of *MEF2C* in the regulation of normal early T-cell development [23]. *MEF2C* was first demonstrated to be highly expressed in immature T-ALL by Homminga et al., 2011 [10], by GEP study. In our study, we found *MEF2C* overexpression to be associated with poor response to prednisolone. This was consistent with previous findings [12]. There is evidence that *MEF2C* dysregulation causes prednisolone resistance by augmenting the anti-apoptotic activity of BCL2 and prednisolone sensitivity can be restored in these cases by BCL2 inhibitor (ABT-737) [22]. We also observed that the median level of *MEF2C* expression was higher in the MRD positive group as compared to the MRD negative group. This may be related to a higher proportion of prednisolone-resistant cases in the high *MEF2C* expression group leading to less clearance of leukemic blasts in this group of patients. Our finding that *MEF2C* high expression was associated with poor EFS and OS, was in contrast with those of [12], who showed that 75% (9/12) of the *MEF2C* deregulated T-ALL patients remained in clinical remission. In our study, 67.9% of *MEF2C* high patients went into clinical remission.

Consistent with a previous report on clinical associations of ETP-ALL [24], we observed it to be associated with poor prednisolone response. This may also be explained by frequent activation of *MEF2C* as a consequence of extended heterozygous 5q deletions that are exclusively observed in ETP-ALL patients. Most 5q deletions also have a deletion of NR3C1, the steroid receptor itself, which contribute to steroid resistance [25]. ETP-ALL was also associated with poorer BM remission rate (78.2% vs. 57.9%; *P* = 0.002) and poor EFS (*P* = 0.007). We did not find any correlation with OS (*P* = 0.42). This contrasts with previous studies [3, 7, 12, 26] and in keeping with a previous study [11].

In conclusion, the three immature T-ALL entities-*MEF2C*-driven, ETP-ALL and especially, ABD-T-ALL, fairly completely characterize the immunophenotypic immature group. Under-representation of immunophenotypically recognized ETP-ALL in *MEF2C*-high, implicitly true transcriptionally defined ETP-ALL, is in keeping with recent observations that diagnosis of ETP-

ALL by immunophenotype underestimates ETP-ALL recognized by gene expression. Seemingly unexpected association of ABD and *MEF2C* high, both markers of immaturity, with non-immature groups-cortical and mature, are explainable in the light of published work and, can be better understood if, in addition to standard work-up, patients are evaluated to allow their classification by the TCR classification scheme that defines developmental stages of T-cells analogous to specific stages in normal T-lymphopoiesis [19]. Our data indicate that while ABD status does not add further to prognostic stratification of T-ALL patients, *MEF2C* expression and ETP-ALL immunophenotype correlate with post-induction MRD positivity, pointing to a need for more studies on the potential utilization of *MEF2C* in addition to the others, as a surrogate for true transcriptionally defined ETP-ALL. Also, non-agreement among the three markers of immature T-ALL-ETP immunophenotype, ABD, and *MEF2C* reflects a cellular complexity in T-ALL that should be investigated further.

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Disclosure of conflict of interest

None.

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