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Original Research

Aberrant overexpression of m6A writer and reader genes in pediatric B-Cell Acute Lymphoblastic Leukemia (B-ALL)

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ABSTRACT

Background: m6A modification, regulated by writers (METTL3, METTL14), erasers (ALKBH5, FTO), and readers (IGF2BPs), is implicated in various cancers, including leukemias.
Methods: In our study, we examined a cohort of 227 pediatric B-ALL patients (152 primary and 75 relapsed) and assessed the expression profiles of m6A machinery genes, including both writers and erasers, as well as the IGF2BP RNA-binding proteins, which are known as m6A readers. We also quantified the absolute percentage of m6A (m6A%). The correlation between m6A machinery gene expression and patient prognosis was studied using univariate and multivariate analyses.
Results: Our analysis revealed a significant upregulation of m6A writers (METTL3 and METTL14), erasers (FTO), and m6A readers (IGF2BPs 1 and 3) in B-ALL patients, both in the primary and relapsed groups. m6A% levels were markedly higher in B-ALL samples than in controls. Multivariate analysis revealed that the expression of IGF2BP3, METTL3, and FTO genes, independently predicted lower overall survival and event-free survival in primary B-ALL patients.
Conclusions: Despite the collective dysregulation of the m6A machinery, the writers and readers appear to have a more dominant phenotype, as evidenced by the significantly elevated m6A% levels. This is the first study to analyze and establish the role of m6A machinery gene expression and its correlation with survival outcomes in a large group of B-ALL patients. These findings could aid in the development of new therapeutics targeting the m6A machinery and help predict relapse in pediatric B-ALL patients.

Introduction

Acute lymphoblastic leukemia (ALL) is the most common leukemia among children. Around 85% of ALL cases belong to the precursor-B-cell immunophenotype (B-ALL), making it the most common subtype across the pediatric as well as the adult population., [1]

Alterations in gene expression play a key role in the transformation of a normal cell to a leukemic cell. RNA-Binding Proteins (RBPs) are known to control gene expression at the post-transcriptional level. Insulin-like growth factor 2 mRNA binding proteins (IGF2BPs) are known oncofetal proteins that play a critical role in the oncogenesis of multiple malignancies like cervical, hepatocellular, breast, glial and other tumors[2]. IGF2BP3 overexpression is associated with a very poor outcome in pancreatic ductal adenocarcinomas[3]. *IGF2BP3* is over-expressed in B-ALL with the highest expression observed in the MLL subtype and *IGF2BP1* is specifically over-expressed in ETV6-RUNX1 translocated B-ALL[4–6].

N-6-methyladenosine (m6A) is the most abundant and reversible modification in mRNA which occurs at RRACH consensus sequence found to be enriched in the 3'-UTRs and stop codons of target mRNAs

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[7]. m6A modification is catalyzed by the m6A writer complex consisting of *METTL3, METTL14* and *WTAP*, with *METTL3* and *METTL14* being the key methyltransferases[8,9]. *WTAP* is the regulatory subunit shown to be essential for the activity of *METTL3* and *METTL14*[8,9]. This m6A modification can be reversed by RNA demethylases (m6A erasers) namely *FTO* and *ALKBH5* that oxidatively reverse m6A to adenosine[8,9] . The cellular fate of m6A-modified mRNA is controlled by the type of reader which interacts with the mRNA. *IGF2BPs* have been identified as novel m6A readers with their binding sites overlapping with the m6A RRACH consensus sequence[10]. *IGF2BPs* are thought to bind mRNA targets which have the m6A mark and subsequently stabilize them.

Emerging evidence suggests that m6A machinery and its alterations play an important role in various cancers. *METTL3* overexpression has been linked to the progression of nasopharyngeal carcinoma and cervical cancer[11,12] while downregulation of *METTL14* was found to be associated with tumor metastasis in hepatocellular carcinoma cells[13].

There are no reports so far that have characterized the m6A status, machinery gene expression and their correlation with clinical parameters in a large cohort of primary and relapsed B-ALL patients. In the current study, we have evaluated the expression levels of m6A machinery genes (writers, erasers and readers) along with the levels of m6A RNA methylation in the bone marrow of primary and relapsed pediatric B-ALL patients. We further estimated the correlation of m6A levels and related gene expression levels on survival outcomes of B-ALL patients.

Methodology

Study design and patient recruitment

This was a prospective observational cohort study, which included pediatric (≤18yrs) patients with de novo (primary) as well as relapsed B-ALL presenting to the medical oncology outpatient clinic of our cancer hospital between October 2018 to June 2022. Children were screened at their outpatient visits and included in the study before the initiation of treatment. Patients with mixed phenotypic acute leukemia, previous history of malignancy, or already ongoing treatment were excluded.

Multiple controls were used for the gene expression and m6A assays: a) Peripheral blood (PB) collected from healthy adult individuals (PB) (n = 20) b) Uninvolved bone marrow (BM) samples from the staging marrow of patients with other malignancies (BM) (n = 18) c)MACS sorted CD19+ cells (n = 6) from PB of healthy adults d) CD34+ Hematopoietic stem cells (HSCs) from leftover mobilised donor marrow. Informed consent was taken from adult healthy controls and parents/ guardians of children <18 years of age and additionally, assent was taken from children >7 years of age after approval by the institute ethics committee (IECPG-267/28.06.2018, RT- 12/18.07.2018) and by Declaration of Helsinki regulations.

Clinical evaluation and treatment protocol

Diagnosis of primary or relapsed B-ALL was established by morphology of bone marrow aspirate along with immunophenotypic confirmation. All primary patients were risk-stratified by karyotyping, and FISH/RT-PCR for common translocations. A cerebrospinal fluid (CSF) cytopathology was done to detect central nervous system involvement as per institutional protocol. Risk stratification and treatment were instituted according to the ICiCLe-ALL-14 protocol[14] for primary patients, while the MRC-UK ALL R1 protocol was followed for relapsed patients[15]. An early relapse is defined as a relapse before 36 months of achieving the first complete remission and relapses after that are considered to be late. Complete remission (CR) was defined as <5% blasts in bone marrow aspirate, while cut-off for minimal residual disease (MRD) by flow cytometry was considered to be 0.01%.

Patient Sample collection and RNA Isolation

After inclusion in the study, bone marrow samples were collected from the patients and controls during routine clinical evaluation before treatment initiation. Peripheral blood samples were collected from healthy adult volunteers.

The baseline clinical and demographic details including age, sex, hemogram findings such as WBC count, platelet count and hemoglobin levels, karyotyping and molecular/ FISH data including details for sentinel translocation such as *ETV6-RUNX1* (t12;21), *TCF3-PBX1* (t1:19), *KMT2A* rearrangement (t4;11) along with *BCR-ABL1* (t9;22), results of FISH, PCR, CSF cytopathology, response details (CR and MRD status) were obtained from medical records. Event-free survival (EFS) was defined as the time from inclusion in the study to the date of relapse or death due to any cause while Overall survival (OS) was defined as the time from inclusion in the study to death due to any cause. The survival data were censored on 25/12/2022.

CD19+ cells were isolated from the peripheral blood of healthy adult individuals using LS MACS columns (Miltenyi Biotech). The bone marrow samples collected from patients (primary and relapse) along with Peripheral blood controls and CD19+ MACS sorted cells, were subjected to RBC lysis with buffer for 30 mins at 4°C.

The samples were centrifuged at 1,500g for 10 minutes at 4°C, and subsequently washed with 1X Phosphate Buffer Saline (Gibco) followed by centrifugation. The pellet obtained was resuspended in 1ml of TRIzol (Thermo Fisher) followed by RNA Isolation. 200µl of chloroform was added per 1ml of TRIzol. The samples were centrifuged at 12,000g for 15 mins at 4°C to allow separation of layers. 0.5ml of Isopropanol (Merck) was added to the aqueous layer and the samples were centrifuged to precipitate the RNA. The pellet obtained was washed with 70% ethanol (Thermo) at 7,500g for 10mins at 4°C. The supernatant was discarded and the pellet was air-dried to remove residual ethanol, dissolved in nuclease-free water (Qiagen) and stored at -80°C till further use.

Real-time PCR for m6A machinery and IGF2BPs

cDNA was synthesised from 500ng of isolated RNA using RevertAid Reverse Transcriptase (Thermo Fisher), Random Decamers (Sigma) and Ribolock (Thermo Fisher). Real-time PCR (qPCR) was done (40 cycles, 95°C for 15s, 60°C for 30s and 72°C for 30s) with SYBR green (Takara) using gene-specific primers for m6A writers (*METTL3, METTL14*), m6A erasers (*FTO, ALKBH5*) and m6A readers (*IGF2BPs-1 &3*) (Table-S4). qPCR reactions were done in triplicates. The relative gene expression was calculated using the delta CT (ΔCt) method. The delta Ct method involves normalising the gene expression of a target gene to a reference gene to analyse the relative gene expression. Relative expression=2^(Ct value of housekeeping gene-Ct value of gene of interest). *RNA polymerase II* was used as the reference gene.

m6A quantification

m6A modification has been reported to be present on 0.1%-0.4% of adenosine residues in cellular RNAs. The abundance of m6A modification (m6A%levels) is less than 0.1% of the total RNA[7,16]. We estimate the proportion of N-6-methylated adenosines present in total RNA using an anti-m6A antibody-based colorimetric kit. It utilises an absorption-based quantification of m6A modification in the total RNA of each sample.This is referred to as m6A%. We have evaluated the percentage of m6A modification in total RNAs of B-ALL patients, primary and relapsed, along with control samples. m6A% levels were quantified in a subset of samples, which included BM of primary (n=44), relapsed (n=41) B-ALL patients and controls (BM n=16, PB n=20, CD34+ HSCs n=5, MACS sorted CD19+ cells (n=6), each containing 200ng of total RNA. We employed an anti-m6A antibody-based RNA methylation quantification kit (colorimetric) (P-9005-96; EPIGENTEK) to estimate the percentage of m6A modification in each sample.

Statistical analysis

Comparison of gene expression and m6A levels in patients and controls

Statistical analysis was done using Prism version 10 (GraphPad Version 10) and SPSS v25.0 (IBM). Descriptive statistics were used to summarise the baseline clinic-demographic details of the primary and relapsed B-ALL patients. The relative gene expression of m6A writers (*METTL3, METTL14*), m6A erasers (*FTO, ALKBH5*) and m6A readers (*IGF2BPs-1 &3)* and m6A levels were evaluated and compared between different patients and control groups using Non-parametric Mann-Whitney tests.

Impact of the expression level of m6A machinery genes and IGF2BPs on survival outcomes

Survival outcomes of the cohort were analyzed using Kaplan-Meier analysis. Multivariate Cox regression analyses in a forward stepwise manner were used to predict the impact of the gene expression level of m6A machinery genes on EFS and OS of both primary and relapsed patient groups.

Impact of clinical factors on survival outcome and independent prognostic impact of m6A machinery genes and IGF2BPs

The impact of clinical and demographic factors, including age, gender, hemoglobin, total leucocyte count, platelets, molecular risk category (good risk and bad risk), karyotype risk category (good risk and bad risk), prednisolone response (primary patients: achievement of CR, MRD status, CNS involvement were used for Cox regression analyses. In relapsed patients, the duration of remission and extra-medullary involvement were also included. It is well known that children younger than 10 years tend to have a better prognosis with lesser chances of relapse in B-ALL. NCI guidelines stratify patients with age more than or equal to 10 years as high-risk[17–20]. The molecular category contains the data regarding the translocation status of the patients obtained using FISH or RT-PCR. Here, the translocation groups-*TCF3-PBX1* and *BCR-ABL1, KMT2A* rearranged B-ALL, which are already known to be associated with poor survival outcome, were taken together as 'poor prognostic molecular category (poor risk)' and *ETV6-RUNX1* along with NKST (no known sentinel translocation) were clubbed in 'good prognostic category (good risk)'[14,17,21–24].

Univariate analyses were conducted, and factors with $p<0.1$ in the univariate analysis were included in a multivariate model in a forward stepwise manner, with factors achieving $p<0.05$ considered significant. A combined multivariate analysis incorporating significant clinical and demographic factors alongside gene expression levels was performed to ascertain their independent prognostic value regarding survival outcomes. The effect of the m6A% level on EFS and OS was evaluated using the chi-squared test. Pearson's chi-squared test was employed to determine significance. When variables had an expected count of less than 5, Fisher's exact test was utilised to assess significance.

Results

Study population

During the study period, a total of 227 (152 primary and 75 relapsed) pediatric B-ALL patients were included. The clinical and demographic factors of the included patients are summarized in Table 1.

The median age of the primary B-ALL cohort was 6 years, with a significant male preponderance (Male: Female = 2:1). For the primary B-ALL cohort, the CR rate was 75.8%. The median overall survival (OS) for patients with primary B-ALL was observed to be 55.6 months, while event-free survival was 34.8 months.

The median age of the relapsed B-ALL cohort was 9 years, with a significant male preponderance (Male: Female = 2:1). Within the relapsed cohort, two-thirds of patients (66.7%) experienced early relapse. The CR rate for re-induction among relapsed patients was 60%.

Table 1
Demographic baseline characteristic features of pediatric newly diagnosed (Treatment primary B-ALL) B-ALL patients (n=152) and relapsed B-ALL patients (n=75).

S. No.	Clinical parameter	Treatment Primary B-ALL (n = 152)	Relapse (n = 75)
1	Age (years) (median with range)	6 (0.6-18)	9 (2-22)
2	Hemoglobin (g/dL) (median with range)	7.1 (1.7-15.20)	8.750 (3.9-14.9)
3	Total Leucocyte Count (X 10^3 cells/μL) (median with range)	22400 (340-3610000)	7565 (600-240500)
4	Platelets (X 10^3 cells/μL) (median with range)	44000 (3300-661000)	43500 (1000-440000)
5	Gender		
	Male	100 (65.8%)	54 (72.0)
	Female	52 (34.2%)	21(28.0)
6	Cytogenetics (Karyotype)		
	Hyperdiploidy	22 (14.5)	11(14.7)
	Hypodiploidy	0	2 (2.7)
	BCR-ABL (t9;22)	2 (1.3)	1 (1.3)
	E2A-PBX1 (t1;19)	1(0.7)	1(1.3)
	Trisomy	1(0.7)	0
	Normal	78 (51.3)	34 (45.3)
	Complex Karyotype	2 (1.3)	0
	Others[#]	2 (1.3)	1 (1.3)
	Failed	18 (11.8)	13 (17.3)
	Not done*	26 (17.1)	12 (16.0)
7	Translocation		
	No Known Sentinel Translocation (NKST)	80 (52.6)	49 (65.3)
	BCR-ABL	12 (7.9)	5 (6.7)
	ETV6-RUNX1	20 (13.2)	12 (16.0)
	TCF3-PBX1	11 (7.2)	4 (5.3)
	KMT2A	5 (3.3)	-
	Not Done**	24 (15.8)	5 (6.7)
8	Day 8 Blasts count		-
	Good responders (<1000blasts)	32 (21.1)	
	Bad responders (≥ 1000 blasts)	82 (53.9)	
	Not available	38 (25.0)	
9	CSF cytopathology		-
	Positive	7 (4.5)	21 (28)
	Negative	121 (79.1)	53 (70.6)
	Not available/Traumatic	24 (15.8)	1 (1.3)
10	CR achieved		-
	Yes	116 (75.8)	45 (60.0)
	No	10 (6.53)	9 (12.0)
	Not available***	26 (17.1)	21 (28.0)
11	Minimal residual disease		
	Negative	82 (53.5)	27 (36.0)
	Positive	39 (25.4)	27 (36.0)
	Not available[##]	31 (20.3)	21 (28.0)
12	Site of Relapse/type of relapse	-	
	Medullary (BM only)		53 (68.0)
	Medullary +Extra medullary (BM+ CNS, BM+CNS± Testes)		21 (32.0)
13	CR duration	-	
	Early relapse (<36months)		50 (66.7)
	Late relapse (≥36months)		24 (32.0)
	Not known		1 (1.3)
14	Median EFS (in months)	34.800 (17.683-51.917)	8.167 (0.03-17.084)
15	Median OS (in months)	55.633 (41.416-69.871)	10.933 (1.417-20.450)

*, ** done but failed, # Down Syndrome, 46 XY, (t1;16) (Q21:Q13) del (3)p23, del (12) P13(18)/ 46XY (2), 45 XY, t (7;9), ## not evaluable, includes patients who died during induction phase, *** includes patients who died during induction and lost to follow up

[1]CSF- Cerebrospinal fluid, CR- Complete Remission, BM- Bone marrow, CNS-Central Nervous System, OS- Overall Survival, EFS-Event-Free Survival

The median overall survival (OS) for relapsed patients was recorded at 10.9 months, while event-free survival was noted to be 8.1 months.

Gene expression of m6A RNA methylation machinery genes

We observed that the relative mRNA expression of m6A writers (*METTL3* and *METTL14*) was significantly higher in B-ALL patients (both primary and relapsed patients) as compared to all controls (Figure 1A and 1B).

Among the B-ALL patient cohorts, relapsed samples showed higher expression of *METTL3* and *METTL14* than primary B-ALL samples (Figure 1A) as well as the BM and PB controls. Interestingly, the sorted CD19+ cells showed a higher expression of *METTL14* than B-ALL patient samples (Figure 1B).

Among the m6A erasers, we observed that the *FTO* mRNA relative gene expression was significantly higher in B-ALL patients (both primary and relapsed) as compared to all controls (BM, PB and CD19+). However, the expression of the *FTO* gene was comparable between primary B-ALL and relapsed patient samples (Figure 1C).

The expression of *ALKBH5* in the primary and relapsed B-ALL samples were comparable to the PB and BM controls. The CD19+ cells showed a slightly higher but significant expression for *ALKBH5* than the primary B-ALL patient samples and PB controls (Figure 1D). *ALKBH5* expression was significantly higher in relapsed samples than in primary samples. The regulatory subunit of m6A writer complex, *WTAP*, showed higher expression in B-ALL patients (both primary and relapsed) than in controls. There was no difference within the primary and relapsed B-ALL samples for *WTAP* expression (Figure 1E).

The mRNA expression of m6A reader *IGF2BP3* was found to be significantly higher in B-ALL patients (primary and relapsed) compared to all controls (Figures 1F and G). The relapsed patient samples showed the highest expression for *IGF2BP3* (Figure 1F).

IGF2BP1 gene is previously known to be specifically overexpressed only in ETV6-RUNX1 translocated B-ALL. *IGF2BP1* expression was higher in B-ALL patient samples which was not statistically significant. The relapsed B-ALL samples showed significantly higher *IGF2BP1* expression than BM and PB controls (Figure 1G).

The ratio of the m6A writers *METTL3/METTL14* to the m6A eraser ALKBH5 was also significantly higher in B-ALL patients (both primary and relapsed) (Figure 2A and 2B). The ratio of the m6A writers *METTL3/METTL14* to the m6A eraser *FTO* showed comparable expression between the B-ALL patients and controls (Figure 2C and 2D).

m6A quantification in B-ALL patients

m6A quantification was done in a subset of B-ALL patient samples and controls. The percentage of m6A level in total RNA (m6A%) was significantly increased in B-ALL patients (primary and relapsed) as compared to controls. The percentage of m6A levels was similar in relapsed B-ALL and primary B-ALL patient groups. Interestingly, the BM controls had a higher m6A% as compared to the other controls, and the CD19+ cells isolated using MACS had very low m6A% levels (Figure 2E).

Correlation of expression of m6A machinery genes and IGF2BPs

The correlation between the gene expression profiles of writers, erasers and readers was analysed in primary and relapsed B-ALL samples. Among the primary B-ALL samples, a significant correlation was observed between the m6A eraser *FTO* and the m6A writer *METTL14* (Spearman's correlation coefficient, $\rho=0.582$, $p\text{-value}<0.001$). All correlations are mentioned in Table S1.

We studied m6A% levels in a small cohort of primary B-ALL samples (n = 44) (Table S2). The primary B-ALL samples had significantly higher expression of m6A machinery genes. The percentage of m6A levels was also seen to be higher in primary B-ALL than in controls. Therefore, we evaluated whether there is a correlation between the percentage of m6A levels and gene expression profiles of the patient samples. We observed that there was an inverse correlation between the m6A levels and the m6A eraser *ALKBH5*($\rho = -0.319$, $p\text{-value}=0.035$). *IGF2BP3* gene expression profile also showed an inverse correlation with the m6A levels in primary B- ALL samples ($\rho= -0.382$, $p\text{-value}=0.011$) (Table S2).

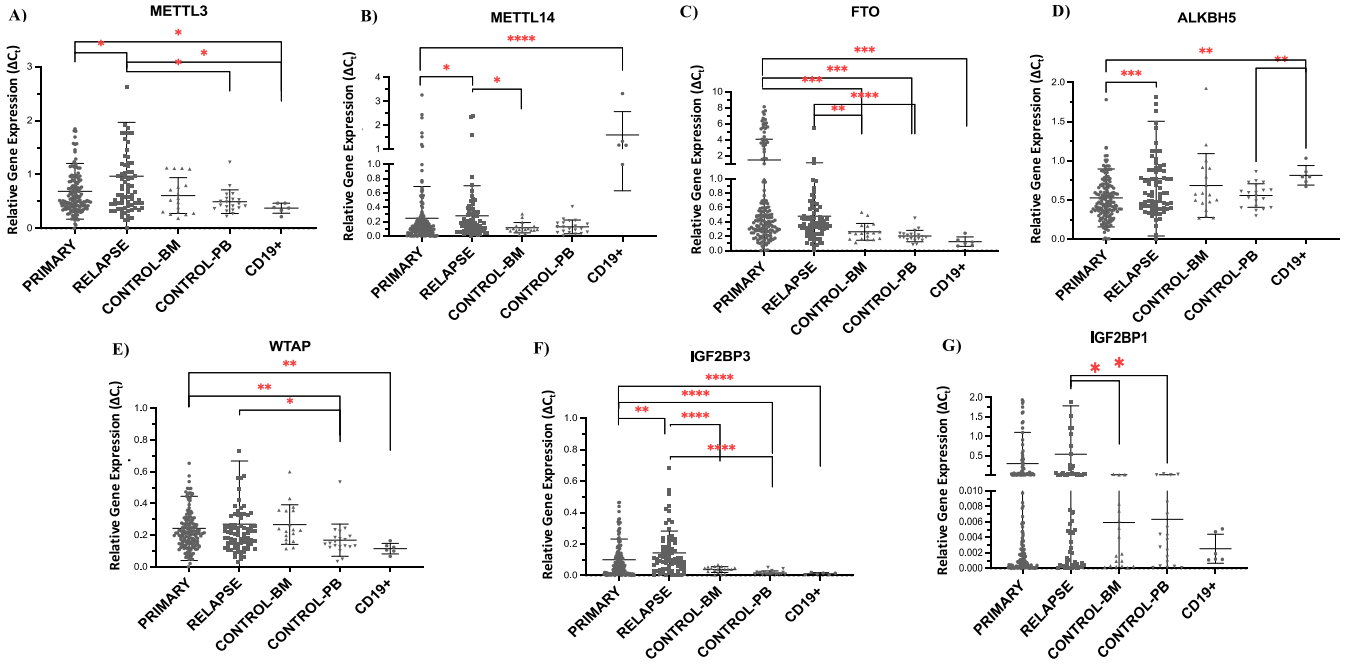

Figure 1. Gene expression of m6A machinery in B-ALL patient samples. The mRNA expression of A) m6A writers *METTL3* B) *METTL14*, m6A erasers C) *FTO* and D) *ALKBH5,* E) *WTAP* m6A readers F) *IGF2BP3* and G) *IGF2BP1* measured by Real-time PCR in B-ALL newly diagnosed (n=152), relapsed (n=75) BM patient samples and controls (BM n=18, PB n=20, CD19+ n=6). Mann-Whitney non-parametric test was used for comparison between the two groups. p-value <0.05 was considered significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

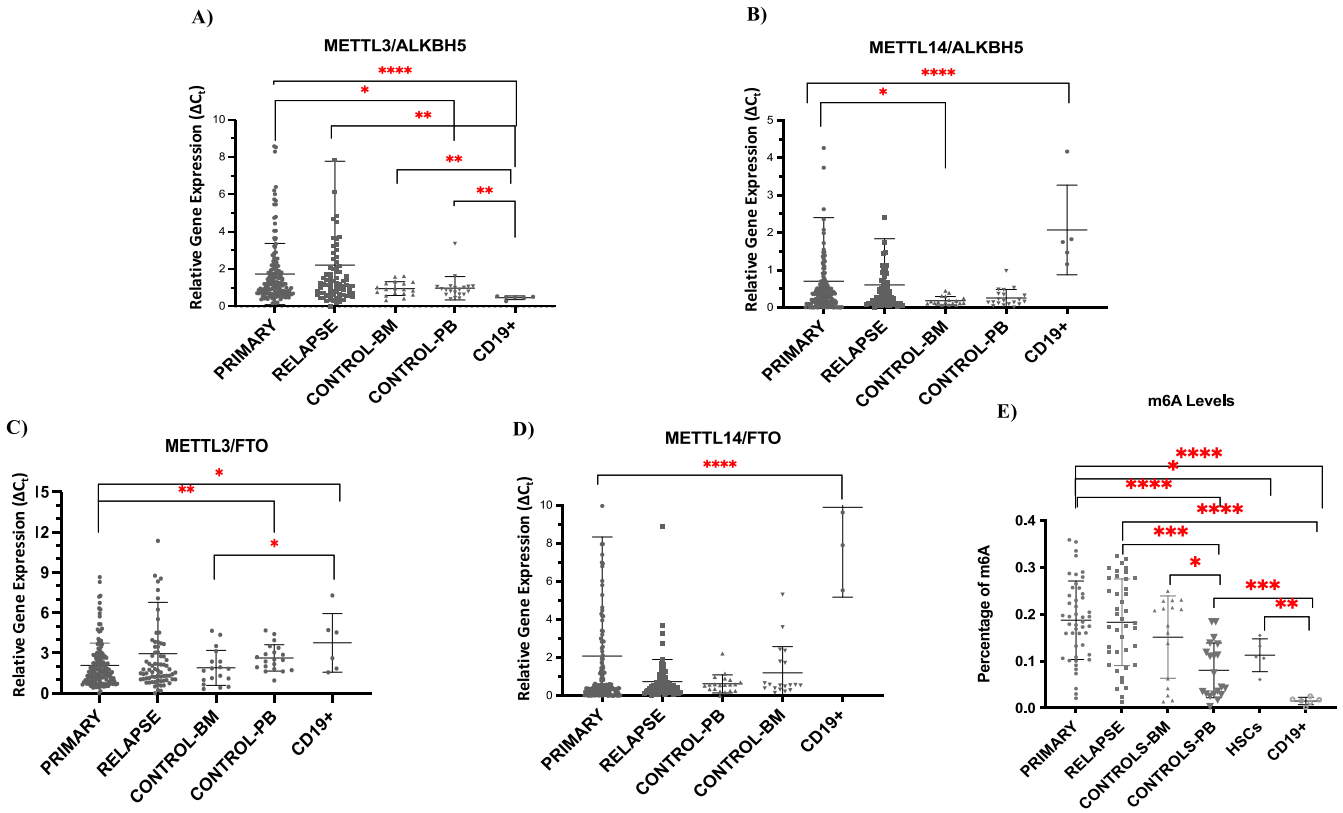

Figure 2. Ratio of m6A writers to erasers and m6A% levels in B-ALL. The mRNA expression of A) Ratio of *METTL3* to *ALKBH5*, B) *METTL14* to *ALKBH5*, C) *METTL3* to *FTO* D) *METTL14* to *FTO* by Real-time PCR in B-ALL primary (n=152), relapsed (n=75) BM patient samples and controls (BM n=18, PB n=20, CD19+ n=6). E) Percentage of m6A in BM of primary B-ALL (new) (n=44), relapsed B-ALL (n=41) patient samples and controls (BM n=16, PB n=20, CD34+ HSCs n=5, CD19+ n=6) Mann-Whitney non-parametric test was used for comparison between two groups. p-value <0.05 was considered significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

In the relapsed samples, we observed that the m6A writer *METTL14* showed a significant correlation with the m6A eraser *FTO* (ρ=*0.426, p-value<0.001*) and the m6A reader- *IGF2BP1* (ρ = *0.510, p-value<0.001*). A significant correlation was observed between the m6A erasers *ALKBH5* and *FTO* (ρ = *0.552, p-value<0.001*). (Table S2). The m6A gene expression profiles did not show a significant correlation with the percentage of m6A levels in relapsed B-ALL samples (Table S2).

Impact of clinical factors on survival outcomes

The median EFS and OS of the primary ALL cohort was 34.8 months (17.7-51.9 months) and 55.6 months (41.4-69.9 months) respectively. On multivariate analyses, age>10 years *(HR= 3.297, p<0.001)* and poor molecular prognostic category *(HR = 2.103, p=0.024)* were predictive of inferior EFS (Figure S1A-B). Similarly, age>10 years *(HR = 3.418, p<0.001)* and poor molecular prognostic category *(HR = 2.468, p = 0.013)* were predictive of inferior OS in primary ALL patients (Figure S2A-B) (Table 2).

For patients with relapsed ALL, the median EFS and OS were 8.1 months (0.03-17.1 months) and 10.9 months (1.4-20.4 months) respectively. Isolated medullary relapse has been shown to have a poorer prognosis than isolated extramedullary or combined medullary and extramedullary relapse[20,25–29].

On multivariate analyses, poor molecular prognostic category *(HR = 4.670 p<0.00)* and isolated medullary relapse *(HR=3.032 p=0.009)* were predictive of inferior EFS (Figure S3 A-C). Similarly, age>10 years *(HR=2.277 p=0.021),* poor molecular prognostic category *(HR = 44.876 p=0.001)* and isolated medullary relapse *(HR = 3.498 p= 0.006)* were predictive of inferior OS in relapsed patients (Figure S4 A-C) (Table 3).

Association of m6A methylation machinery gene expression profiles with survival outcome of B-ALL patients

On multivariate Cox regression analyses, the relative gene expression of *IGF2BP3* (*HR = 7.848 p= 0.007*), *METTL3* (*HR = 1.772 p-value = 0.011*) and *FTO* (*HR = 1.037 p= 0.002*) were predictive of inferior EFS for primary B-ALL samples. Similarly, relative gene expression of *IGF2BP3* (*HR = 6.876 p = 0.016*), *METTL3 (HR = 1.668 p= 0.030)* and *FTO* (*HR =1.036 p = 0.001*) were predictive of inferior OS (Table 4A).

On combining significant clinical factors and gene expression, only gene expression of *FTO* was independently predictive of inferior EFS (*HR =1.044 p= 0.001*) and OS (*HR=1.039 p=0.001*). The ratios of m6A writers to m6A erasers were not predictive of survival (Table S1).

Among the relapsed patients, the expression profile or fold change of none of the m6A machinery genes was predictive of EFS and OS. However, on exploratory analyses, the *METTL3/ALKBH5* gene expression ratio was predictive of inferior OS *(HR =1.052 p= 0.032)* and showed a trend towards inferior EFS *(HR =1.044 p = 0.066)* (Table 4B). As already mentioned, all the relapsed ALL patients had an extremely poor prognosis.

Correlation of methylation with gene expression profiles of m6A machinery along with clinical parameters

We evaluated the association between the percentage of m6A levels, and studied it along with the clinical parameters, in primary B-ALL (n = 44) and relapsed (n = 41) B-ALL patient samples. This correlation was studied for only those patients whose m6A% levels had been evaluated. The B-ALL patients (primary B-ALL and relapse) were categorized into m6A high and low depending upon the median m6A% levels value

Table 2
Correlation of clinical parameters with survival (EFS and OS) using univariate and multivariate analysis in primary (treatment naïve) B-ALL patients (n=152).

S. No.	Parameters		Median (months)	Event-free survival (EFS)				Overall Survival (OS)			
				Univariate		Multivariate		Univariate		Multivariate	
				HR 95% CI	*p-value*	HR 95% CI	*p-value*	HR 95% CI	*p-value*	HR 95% CI	*p-value*
1	Age (n = 152)	<10 (101)	55.63	1		1		1		1	
		≥ 10 (51)	13.96	3.007 (1.809-4.999)	<0.001	3.297 (1.786-6.088)	<0.001	2.993 (1.735-5.163)	<0.001	3.418 (1.725-6.773)	<0.001
2	Gender (n = 152)	Male (100)	32.7	0.943 (0.562-1.580)	0.822			0.967 (0.553-1.691)	0.906		
		Female (52)	55.63	1				1			
3	Cytogenetics (n = 106)	Good Risk = 101	55.63	1				0.899 (0.122-6.645)	0.917		
		Bad Risk = 5*	23.56	2.91 (0.881-9.619)	0.08			1			
4	Molecular Category (Translocation) (n = 127)	Good Risk = 99	55.63	1		1		1		1	
		Bad Risk = 28	20.26	2.061 (1.127-3.771)	0.019	2.103 (1.103-4.007)	0.024	2.336 (1.207-4.521)	0.012	2.468 (1.206-5.050)	0.013
5	TLC (n = 151)	<50000 = 99	55.63	1				1			
		≥ 50000-52	25.26	0.627 (0.381-1.032)	0.066			0.682 (0.397-1.172)	0.166		
6	HB (n = 151)	≤8 = 97	39.93	1				1			
		>8 = 54	31.96	0.977 (0.585-1.630)	0.928			0.654 (0.384-1.117)	0.12		
7	Platelets (n = 151)	<50000 = 84	40.16	1				1			
		≥ 50000 = 67	32.7	1.024 (0.624-1.680)	0.926			1.088 (0.641-1.848)	0.755		
8	MRD (n = 115)	Negative = 78	NR	1				1			
		Positive = 37	39.93	1.522 (0.811-2.857)	0.191			1.507 (0.754-3.013)	0.246		
9	CR (n = 126)	Yes = 115	59.4	1				1			
		No = 11	13.7	2.365 (1.053-5.310)	0.037			2.421 (1.007-5.818)	0.048		
10	Day 8 Blasts (n = 114)	<1000 = 82	55.63	1				1			
		>1000 = 32	40.16	1.07 (0.553-2.068)	0.841			0.942 (0.451-1.967)	0.874		
11	CSF (n = 127)	Positive = 9	40.16	0.902 (0.476-1.709)	0.751			1.114 (0.343-3.620)	0.858		
		Negative = 118	55.63	1				1			
12	Risk Category (n = 146)	HR = 65	28.13	2.167 (0.992-4.734)	0.052			2.129 (0.941-4.814)	0.07		
		IR = 38	31.96	2.165 (1.094-4.287)	0.027			1.857 (0.895-3.855)	0.097		
		SR = 43	NR	1				1			

* Between cytogenetics risk category and molecular risk category, molecular risk category was taken for multivariable analyses as both parameters are collinear and cytogenetics data was not available/failed for 44 patients (28.9%)

obtained. The median EFS and OS of patients with low m6A levels were not significantly different than those with high m6A levels (Table S3).

In the relapsed cohort, the median EFS and OS of patients with high m6A levels were lower than those with high m6A levels (Median EFS with m6A high: 6.767 vs 30.533 m in m6A low; *p=0.26*; Median OS with m6A high 10.433 m vs 6.133 m in m6A low; *p=0.314*). However, the difference in EFS and OS could not be demonstrated due to the small sample size.

The hemoglobin levels correlated significantly with m6A levels in primary B-ALL patients *(p=0.021)* (Table S3). We did not find any clinical parameter to significantly correlate with m6A levels in relapsed B-ALL patients (Table S3).

Discussion

In this study, we observed that the expression of m6A writers METTL3 and METTL14, the m6A eraser FTO, and the readers IGF2BP3 and IGF2BP1 was significantly elevated in pediatric B-ALL patient samples. Elevated METTL3 expression has been implicated in multiple cancers, promoting the translation of oncogenic mRNAs such as EGFR

Table 3
Correlation of clinical parameters with survival (EFS and OS) using univariate and multivariate analysis in B-ALL relapsed patients (n=75).

S. No.	Parameters		Median (months)	Event-free survival (EFS)				Overall Survival (OS)			
				Univariate		Multivariate		Univariate		Multivariate	
				HR 95% CI	*p-value*	HR 95% CI	*p-value*	HR 95% CI	*p-value*	HR 95% CI	*p-value*
1	Age (n = 75)	<10 years = 49	30.533	1				1		1	
		≥ 10 years = 26	2.6	2.358 (1.230-4.521)	0.01			2.63 (1.362-5.078)	0.004	2.277 (1.131-4.585)	0.021
2	Gender (n = 75)	Male = 54	8.167	0.967 (0.467-2.000)	0.927			1.188 (0.560-2.520)	0.653		
		Female = 21	13.067	1				1			
3	Cytogenetics (n = 50)	Good Risk = 45	8.167	1				1			
		Bad Risk = 5	16.1	0.85 (0.253-2.851)	0.792			0.889 (0.266-2.970)	0.849		
4	Molecular Category (Translocation) (n = 70)	Good Risk = 61	16.1	1		1		1		1	
		Bad Risk = 9	2.467	4.152 (1.748-9.860)	0.001	4.67 (1.966-11.092)	<0.001	4.495 (1.874-10.779)	0.001	4.876 (1.960-12.131)	0.001
5	TLC (n = 74)	<11000 = 27	16.1	1				1			
		≥ 11000 = 47	8.167	0.945 (0.481-1.859)	0.871			1.01 (0.511-1.996)	0.977		
6	HB (n = 73)	≤8 = 30	6.767	1.656 (0.866-3.166)	0.127			1.883 (0.973-3.642)	0.06		
		>8 = 44	NR	1				1			
7	Platelets (n = 74)	<50000 = 39	6.767	1.538 (0.796-2.970)	0.2			1.35 (0.695-2.621)	0.376		
		≥ 50000 = 35	NR	1				1			
8	MRD (n = 54)	Negative = 27	NR	1				1			
		Positive = 27	30.533	0.939 (0.397-2.219)	0.886			0.826 (0.342-1.998)	0.672		
9	CR Duration (n = 74)	<36 months = 50	5.933	0.555 (0.261-1.180)	0.126			1.644 (0.774-3.494)	0.196		
		≥ 36 months = 24	NR	1				1			
10	Site of Relapse (n = 75)	Medullary = 51	6.767	2.765 (1.206-6.339)	0.016	3.032 (1.318-6.976)	0.009	2.585 (1.1.26-5.932)	0.025	3.498 (1.430-8.558)	0.006
		Medullary +Extra-medullary = 24	NR	1		1		1		1	
11	CR (n = 54)	No = 9	5.1	1.357 (0.389-4.732)	0.632			0.712 (0.161-3.148)	0.655		
		Yes = 45	30.533	1				1			

and TAZ in lung cancer[30], and contributing to hepatocellular carcinoma progression[31]. In acute myeloid leukemia (AML), METTL3 enhances the translation of cMYC, BCL2, and PTEN, leading to reduced differentiation and apoptosis[32]. In chronic lymphoblastic leukemia (CLL), METTL3 induces expression of splicing factors SF2B1, SF3A1, and SF3BA3, in an m6A-dependent manner supporting cell growth *in-vitro* and *in-vivo*[33]. We have also observed overexpression of *METTL3* in our sample cohorts suggesting a role for *METTL3* in the pathogenesis of B-ALL.

METTL14, a pseudo-methyltransferase, facilitates METTL3-mediated m6A methylation and has been linked to AML progression by regulating MYB and MYC expression[34]. In pancreatic cancer, METTL14 overexpression inhibits the translation of tumor suppressor PERP mRNA [35], while in breast cancer, LINC0092 recruits METTL14 to stabilize CXCR4 and CYP1B1, enhancing tumor growth and development of breast cancer[36]. Our study also identified METTL14 overexpression in B-ALL.

Interestingly, the RNA demethylases (m6A erasers) -*FTO* and *ALKBH5*- have also been shown to play a significant role in cancer. In our data, *FTO* demethylase is significantly overexpressed in B-ALL patients. *FTO* was found to be aberrantly overexpressed in AML subtypes such as carrying t(11q23)/MLL and/or NPM1 mutations. FTO inhibits AML cell differentiation by reducing m6A levels on ASB2 and RARA[37] and is associated with poor prognosis in cervical squamous cell carcinoma, glioblastoma (GBM)[38,39], gastric[40,41], osteosarcoma[42], pancreatic[43], and esophageal cancers[44]. Genome-wide studies further correlate FTO overexpression with poor prognosis in thyroid carcinoma[45]. FTO reduces m6A levels in tumor suppressor genes, decreasing their stability and contributing to oncogenesis.

ALKBH5 plays an oncogenic role in both AML and GBM via the self-renewal of the relevant CSCs/GSCs[46,47]. *ALKBH5* demethylates the nascent transcripts of *FOXM1* and upregulates *FOXM1* expression, which contributes to GSC tumorigenesis[46]. In AML, *ALKBH5* regulates the self-renewal of LSCs by regulating the stability of *TACC3* and *AXL* genes in a *YTHDF2*-dependent manner[47,48]. However, we did not observe upregulation of the *ALKBH5* gene in our sample cohort.

IGF2BPs, oncofetal RNA-binding proteins, are overexpressed in colorectal and breast cancers[2]. IGF2BP3 is elevated in MLL-translocated B-ALL, while IGF2BP1 is overexpressed in ETV6-RUNX1-translocated B-ALL[4–6,49]. As m6A readers, IGF2BPs

Table 4
Correlation of m6A RNA methylation associated gene profiles with survival outcome (EFS and OS) in primary (n=152) and relapsed B-ALL patients (n=75).

S. No.	PRIMARY	Event-free survival (EFS)				Overall survival (OS)			
		Univariate		Multivariate		Univariate		Multivariate	
	Gene Name	HR (95%CI)	*p-value*	HR (95%CI)	*p-value*	HR (95%CI)	*p-value*	HR (95%CI)	*p-value*
1	METTL3	1.554 (0.970-2.489)	0.067	1.482 (0.903-2.433)	0.396	1.482 (0.903-2.433)	0.396	1.668 (1.052-2.646)	0.03
2	METTL14	1.046 (0.547-1.998)	0.893	1.113 (0.579-2.140)	0.749	1.113 (0.579-2.140)	0.749		
3	ALKBH5	1.034 (0.919-1.164)	0.579	1.047 (0.929-1.181)	0.451	1.047 (0.929-1.181)	0.451		
4	FTO	1.032 (1.011-1.054)	0.003	1.032 (1.011-1.052)	0.002	1.032 (1.011-1.052)	0.002	1.036 (1.014-1.059)	0.001
5	IGF2BP1	0.726 (0.461-1.145)	0.169	0.681 (0.398-1.167)	0.162	0.681 (0.398-1.167)	0.162		
6	IGF2BP3	7.253 (1.687-31.179)	0.008	6.728 (1.420-31.876)	0.016	6.728 (1.420-31.876)	0.016	6.876 (1.432-33.002)	0.016
7	WTAP	1.791 (0.584-5.495)	0.308	2.161 (0.810-5.762)	0.124	2.161 (0.810-5.762)	0.124		
S. No.	RELAPSE	Event-free survival (EFS)				Overall survival (OS)			
		Univariate		Multivariate		Univariate		Multivariate	
	Gene Name	HR (95%CI)	*p-value*	HR (95%CI)	*p-value*	HR (95%CI)	*p-value*	HR (95%CI)	*p-value*
1	METTL3	0.966 (0.700-1.335)	0.835	-		0.992 (0.720-1.366)	0.96	-	
2	METTL14	0.977 (0.393-2.425)	0.96	-		1.089 (0.456-2.599)	0.847	-	
3	ALKBH5	1.213 (0.836-1.760)	0.308	-		1.201 (0.827-1.744)	0.337	-	
4	FTO	1.158 (0.805-1.666)	0.428	-		1.18 (0.818-1.703)	0.376	-	
5	IGF2BP1	1.005 (0.951-1.063)	0.852	-		1.01 (0.957-1.065)	0.729	-	
6	IGF2BP3	2.158 (0.187-24.895)	0.537	-		3.323 (0.314-35.195)	0.319	-	
7	WTAP	1.119 (0.575-2.180)	0.119	-		1.158 (0.595-2.253)	0.666	-	

stabilize target mRNAs to enhance translation in an m6A-dependent manner[10]. IGF2BP3 is linked to poor AML prognosis, where it stabilizes RCC2 by binding to its m6A-modified sites[50]. Our findings highlight the oncogenic role of IGF2BP1 and IGF2BP3 as m6A readers in B-ALL.

In this study, we observed that the ratio of m6A writers METTL3/METTL14 to the eraser ALKBH5 was significantly higher in B-ALL samples, indicating increased m6A writer activity. m6A assay confirmed elevated m6A% levels in B-ALL samples compared to controls, marking the first study to demonstrate increased m6A% levels in ALL patient samples. Despite FTO overexpression, significantly higher m6A reader expression and m6A levels suggest that writers, readers, and m6A-modified genes contribute to B-ALL pathogenesis and progression.

Considering the dysregulation of m6A machinery genes seen in B-ALL patients, we analysed the correlation between survival parameters and the gene expression of the m6A machinery genes. Survival analyses in primary B-ALL samples revealed that METTL3, IGF2BP3, and FTO were associated with poor event-free survival (EFS) and overall survival (OS). IGF2BP3 had the highest hazard ratio for OS/EFS, while FTO expression was the strongest predictor of poor survival when combined with key clinical factors such as age and molecular subtype. However, in relapsed B-ALL samples, m6A machinery gene expression did not correlate with survival, suggesting additional contributing factors. An alternative hypothesis is that relapsed B-ALL represents an inherently aggressive, high-risk population with poor chemotherapy tolerance, leading to uniformly upregulated m6A machinery genes without survival correlation.

Correlation analysis revealed a positive feedback loop between METTL14, IGF2BP1, and FTO. FTO expression positively correlated with METTL14 in both primary and relapsed B-ALL (ρ=0.582, 0.426, $p<0.001$), while METTL14 correlated with IGF2BP1 in relapsed B-ALL ($p<0.001$). This aligns with studies by Panneerdoss et., al. (2018), which demonstrated crosstalk between METTL14, ALKBH5, and YTHDF3, influencing mRNA stability in breast cancer by modulating TGFβ1, CCND1, and SMAD3 in m6A-dependent manner[51].

To summarize, our data indicate that overexpression of m6A writers METTL3 and METTL14 increases m6A levels in leukemic cells, contributing to pediatric leukemia development and recurrence. This upregulation, combined with elevated IGF2BP3 and IGF2BP1, stabilizes oncogenic mRNAs and enhances their translation. These findings highlight the dysregulation of m6A machinery in B-ALL as a potential therapeutic target.

Ethics approval and consent to participate

Informed consent was taken from adult healthy controls and parents/guardians of children <18 years of age and additionally, assent was taken from children >7 years of age after approval by the institute ethics committee (IECPG-267/28.06.2018, RT- 12/18.07.2018) and by the Declaration of Helsinki regulations.

Consent for publication

All authors have read the manuscript and agree to publish.

Availability of data and materials

Data sharing does not apply to this article as no datasets were

generated or analysed during the current study.

CRediT authorship contribution statement

Sumedha Saluja: Writing – review & editing, Writing – original draft, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation. **Shuvadeep Ganguly:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Jay Singh:** Writing – review & editing, Resources, Formal analysis, Data curation. **Ayushi Jain:** Resources, Methodology, Formal analysis, Data curation. **Gunjan Sharma:** Writing – review & editing, Validation, Resources, Methodology, Data curation. **Shilpi Chaudhary:** Writing – review & editing, Resources, Formal analysis, Data curation. **Karthikeyan Pethusamy:** Resources, Formal analysis, Data curation. **Parthaprasad Chattopadhyay:** Supervision, Resources. **Anita Chopra:** Writing – review & editing, Resources, Project administration, Formal analysis, Data curation. **Archna Singh:** Writing – review & editing, Resources, Project administration. **Subhradip Karmakar:** Resources, Investigation. **Sameer Bakhshi:** Writing – review & editing, Supervision, Resources, Project administration. **Jayanth Kumar Palanichamy:** Writing – review & editing, Writing – original draft, Project administration, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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