BOHR Journal of Cancer Research
2023, Vol. 1, No. 2, pp. 47–55
DOI: 10.54646/bjcr.2023.10
www.bohrpub.com

RESEARCH

Methylomic change in TET1 and TET2 promoters is increased in children with acute lymphocytic leukemia

Rofaida Refaai[1], Dina Ragab[2], Adel A. Guirgis[1] and Hany Khalil[1]*

[1]Department of Molecular Biology, Genetic Engineering and Biotechnology Research Institute, University of Sadat City, El Sadat City, Egypt
[2]Department of Clinical Pathology, Faculty of Medicine, Ain Shams University, Cairo, Egypt

***Correspondence:**
Hany Khalil,
hkhalil74@gmail.com

Received: 28 November 2023; **Accepted:** 15 December 2023; **Published:** 29 December 2023

Objective: Methylomic changes have been recognized as essential contributors to the development of acute lymphocytic leukemia (ALL). While ALL has traditionally been considered a genetic disease, the increasing importance of epigenetic alterations in leukemogenesis has become evident. Here, our goal was to investigate the changes of DNA methylation level in ALL.
Methods: To assess the DNA methylation pattern in ALL, we quantified gene expression of the methylation-related factors, including DNMTs, methionine synthase (MS), and the demethylation factors, TET gene family using qRT-PCR.
Results: The expression pattern of DNMTs, including DNMT1, DNMT3a, and DNMT3b, was markedly upregulated in samples derived from patients. On the contrary, the expression of the methyl creator, methionine synthase (MTR gene) was down-regulated in samples derived from children with ALL which revealed low concentration of vitamin B12 in their samples. The reduced expression of MTR in children with ALL reveals the disturbance of homocysteine and DNA methylation. Moreover, we examined the expression patterns of TET1, TET2, and TET3 genes implicated in the demethylation process. We found an evident decreasing level of TET1 and TET2 expression in ALL that may refer to an increasing DNA methylation activity in the promoter of the TET gene family.
Conclusion: These data suggest the possible alteration in methyaltion-related factors due to methylomic changes within the promoter of TET1 and TET2 genes.

Keywords: acute lymphocytic leukemia, DNA methylation, DNA methyltransferase (DNMTs), methionine synthase (MS), and ten-eleven translocations (TET)

1. Introduction

Acute lymphocytic leukemia (ALL) is the most common malignancy in children and adolescents and is responsible for a third of childhood cancer deaths. It originates from the malignant conversion of bone marrow progenitor B- and T-cells into leukemic cells, while normal hematopoietic cell development requires tightly controlled regulation of epigenetic modifications (1). Epigenetics was initially definite as heritable modification in gene expression parallel with other variations in genomic sequence. Usually, these genetic alterations are developed through differentiation processes. The stability of these alterations is sustained during the multiple cell cycles, facilitating the new distinct identities of the cells with the same genetic information (2). Methylomic changes play an essential role in modulating gene expression via various interactions such as DNA methylation, nucleosome positioning, miRNAs, and RNA-associated silencing. Besides, DNA methylation reveals the methylation activity within a DNA sequence at CpG-rich sites throughout the genome. Hypermethylation of CpG-enriched gene promoters is ended by transcriptional

CC BY NC ND

suppression of tumor suppressor genes (3). Indeed, DNA methylation alters a specific sequence of genomic DNA leading to useless proteins implicated in aggressive hematological malignancies, specifically in ALL (4). DNA methylation is mediated by DNA methyltransferases, critical enzymes activating DNA methylation. Three active DNMT enzymes (DNMT1, DNMT3a, and DNMT3b) are responsible for transferring methyl groups from S-adenosylmethionine (SAM) to cytosine (5, 6). Vitamin B12 is an animal source and one of the most prominent factors in DNA methylation. It is essential for DNA synthesis and critical in the epigenetic process (7). As a methyl donor, it participates in the monocarbonic acid metabolic pathway, which is particularly significant during carcinogenesis (8).

Notably, Vitamin B12, along with other methyl donors such as folate, serves as a coenzyme for methyltransferases and facilitates the remethylation of homocysteine by methionine synthase through methylcobalamin to methionine (9, 10). Furthermore, vitamin B12 is responsible for converting methyl-THF to tetrahydrofolate (THF). B12, methionine synthase (MS), and methyl-THF are crucial effectors in the transferring of homocysteine to methionine to supply the methyl groups in methylation progress, forming 5-methylcytosine. While DNMT1 methylates newly generate CpG dinucleotides to preserve DNA methylation during replication, DNMT3a and DNMT3b primarily establish methylation patterns, in addition to their role of repairing errors introduced by DNMT1 during DNA replication (11). On the contrary, demethylation activity requires the function of protein family known as Ten-Eleven translocation proteins (TET1, TET2, and TET3). TET proteins are a kind of dioxygenase family that is able to oxidize methylated cytosine (12). TET proteins regulate the methylation levels of 5-cytosine in balancing with DNMTs level (13). Meanwhile, the regulation of the content of 5-hmC in CpG islands has a wide range of vital roles in cancer development or suppression (14). TET proteins have an iron and α-ketoglutarate-dependent oxygenase class, required for their functional progress (15). Excess iron sometimes plays a destructive role of stimulating the secretion of reactive oxygen species, causing oxidative toxicity and facilitating carcinogenesis. Therefore, iron must tightly control intracellular iron homeostasis by iron storage protein ferritin, which helps regulate iron balance within cells, ensuring it is neither deficient nor excessive (16). Moreover, Ferritin, involved in iron metabolism, has been associated with cancer progression, including cell immortalization, avoiding apoptosis, angiogenesis, metastasis, and DNA demethylation (17). Ferritin levels directly relate to p53 protein production and reduce oxidative stress by controlling cellular labile iron content. The tumor suppressor p53 is activated in response to iron deficiency and regulates reactive oxygen species levels (18).

In the current work, we are investigating the expression pattern of different effectors associated with the methylmoic alterations in children with ALL, including *DNMT*s, *MTR, TET1, TET2*, and *TET3*.

2. Methodology

2.1. Ethical issues and samples condition

The current work was approved by the ethical committee of the Faculty of Medicine (Ain Shams University, Egypt) and Genetic Engineering and Biotechnology Research Institute (University of Sadat City, Egypt) (reference number 202010156). The study was carried out from May 2020 to March 2022 with the exclusion criteria including aged patients and children diagnosed with diabetes mellitus and/or kidney failure. Fifty blood samples were taken from children with ALL, while other 50 blood samples were taken from healthy children, which served as control.

2.2. Biochemical analysis

The concentrations of vitamin B12, ferritin, and folate in derivative samples were achieved using vitamin B12 Gen II Elecsys Cobas (Ref. No: 07212771190) and Folate Gen III Elecsys Cobas (Ref. No: 07559992190), respectively (19). The derived blood samples were taken in a trace-element tube (Becton Dickinson) and were left to coagulate. Then 15 μl from the sample was incubated with pretreatment reagents of vitamin B12 for 9 min to measure the concentration of vitamin B12. Then, the ruthenium-labeled vitamin B12 binding effector was appended to each sample for another 9 min, and then streptavidin-coated microparticles and vitamin B12-biotin-labeled reagent were added to each sample and incubated for another 9 min. The samples were then relocated into a measuring tube, and a photomultiplier was used to measure the induced chemiluminescent emission. The final concentration of vitamin B12 was calculated according to the values indicated by the normal curve supplied by the reagents barcode. The folate concentration in derived samples was assessed using the previously described protocol (20).

2.3. Genomic isolation and methylation activities

The genomic DNA was purified from blood samples derived from ALL patients and healthy children using a DNA isolation kit (Qiagen, USA). The methylmoic change was investigated within the promoter region of TET1, TET2, and TET3 in all provided samples using sodium bisulfite-converted protocol. Accordingly, a total of 0.5 mg DNA isolated from derived samples was denaturized and treated with one molar sodium bisulfite

using a DNA modification kit (Methylamp^{TM} DNA Modification kit; Epigentek, NewYork, NY, USA). The PCR product was then amplified using qRT-PCR and the following specific primers to obtain the methylation levels within the promoter region of the TET gene family; TET1-UTR-F-5′-actccctgaggtctgtcctggga-3, TET1-UTR-R-5′ggatcgagacatagctacagagt-3′, TET2-UTR-F-5′- atgg aacaggacagaaccacccat-3′, TET2-UTR-R-5′- atggagcccagag agagatggttca-3′, TET3-UTR-F-5′-cggacgccttcattgctgctgctt-3′, and TET3-UTR-R-5′-taggtgctggggcagaaccacagt-3′. The thermocycling parameters were 95°C for 10 min, and 40 cycles (95°C for 30 sec, 60°C for 30 sec, and 72°C for 45 sec) (21, 22).

2.4. Quantitative RT-PCR procedure

Total RNA was extracted from derived samples using TriZol (Invitrogen, USA), chloroform, and isopropanol protocol. The extracted RNA was purified using an RNA purification kit (Invitrogen, USA) according to the manufacturer's protocol. The purified RNA was used to synthesize the cDNA using the QuantiTech R.T. Kit (Qiagen, USA). The relative expression of DNMT1, DNMT2, DNMT3a, DNMT3b, MTR, TET1, TET2, and TET3 was quantified in patient samples and normalized to their expression in control samples using specific primers (**Table 1**). The SYBR green PCR Kit (Qiagen, USA) was used to amplify the targeted fragments in each indicated gene, in addition to the housekeeping- GAPDH, which served as internal control. The following parameters were used in the PCR machine: 94°C for 5 min, 40 cycles (94°C for 30 sec, 60°C for 30 sec, 72°C for 45 sec). Based on the normalization of cycle threshold (Ct) value for each gene to GAPDH and normalization between samples and control, the delta-delta Ct analysis method was used to achieve the quantification of gene expression presented as fold changes (23, 24).

TABLE 1 | Primer sequences specific for indicated genes to detect their relative expression pattern.

Description	Primer sequences 5′-3′
Forward_DNMT1	CCCATGCATAGGTTCACTTCCTTC
Reverse_DNMT1	TGGCTTCGTCGTAACTCTCTACCT
Forward_DNMT2	CATACAATGCCCGTGTGAGTTCTTAAGG
Reverse _DNMT2	CGTGTGTCTAAATGGCTTGAGTACAGT
Forward_DNMT3	TGCAATGACCTCTCCATTGTCAAC
Reverse_DNMT3	GGTAGAACTCAAAGAAGAGGCGG
Forward _MS	GAGATGCCTGAGACACCCA
Reverse_MS	GTGCACCAGTTTTCGTTCCT
Forward_TET1	GCACGATGCACCTGTACGAT
Reverse_TET1	CACCAAGCTTTTTTGCTGTGAGT
Forward_TET2	GCTCCTGGTGATGGCTACTG
Reverse_TET2	TGTTTGCAGAGGTGAGTGGT
Forward_TET3	GAAGAGCTCCCAAATTGCCT
Reverse_TET3	GCTACAACATGAGCTACTGGC
Forward_GAPDH	TGGCATTGTGGAAGGGCTCA
Reverse_GAPDH	TGGATGCAGGGATGATGTTCT

3. Results

3.1. The deficiency of folate, vitamin B12, and serum ferritin is observed in patients with ALL

A total of 50 children with ALL were involved in the study to monitor the level of hemoglobin, ferritin, folate, and vitamin B12 in isolated serum. As presented in **Table 2**, the frequency of anemic cases within the patients with ALL was 94%, as the children who showed a lacking level of hemoglobin were 47 out of 50. Further investigation of samples derived from these children also showed increased homocysteine in parallel with low ferritin, vitamin B12, and saturated transferrin. Likewise, about 90% of samples derived from children with ALL showed depleted levels of serum folate (less than 140 ng/L), while only 10% of children with ALL showed normal folate levels in their samples. These analyses suggested that the most of children with ALL represented accumulated levels of homocysteine parallel with a significant depletion of hemoglobin, ferritin, and vitamin B12 in their derived serum.

3.2. The relative expression of DNMTs is markedly increased in children with ALL

The potential methylomic changes of genomic DNA were first investigated in this study by analyzing the expression of DNA methyltransferases (DNMTs) in correlation with analyzed vitamin B12 and ferritin. The study utilized qRT-PCR to quantify the expression of DNMTs in both the control group and ALL patients. Interestingly, our findings expose the significant overexpression of DNMT1, DNMT3a, and DNMT3b genes in patients with ALL compared with healthy individuals (**Figure 1**). Particularly, the gene expression pattern of DNMT1 was significantly upregulated and reached three times more in derived samples from ALL patients when compared with samples obtained from healthy children (**Figure 1A** and **Table 3**). Meanwhile, the expression profile of DNMT2 showed negligible differentiation between both derived samples (**Figure 1B** and **Table 4**). Notably, the relative gene expression of DNMT3a increased by more than four-fold change in ALL patients (**Figure 1C** and **Table 5**), while the expression of DNMT3b increased more than five-fold change in patients with ALL compared with healthy ones (**Figure 1D** and **Table 6**). Taken together, these data indicate the overexpression of epigenetics-related factors such as DNMT1 and DNMT3 in samples obtained from children

TABLE 2 | Variables of hemoglobin, vitamin B12, ferritin, and the other factors in derived samples from children with ALL compared with healthy individuals.

Cases	Hemoglobin less than 1 mg/dL		Ferritin less than 300 ug/L		Saturated transferrin less than 20%		Vitamin B12 less than 200 ug/L		Folate less than 140 ng/ml		Homocysteine more than 15 pcmol/L	
	No	%	No	%	No	%	No	%	No	%	No	%
Healthy $n = 50$	8	16%	7	14%	5	10%	6	12%	7	14%	6	12%
ALL $n = 50$	47	94%	48	96%	45	90%	48	96%	45	90%	44	88%

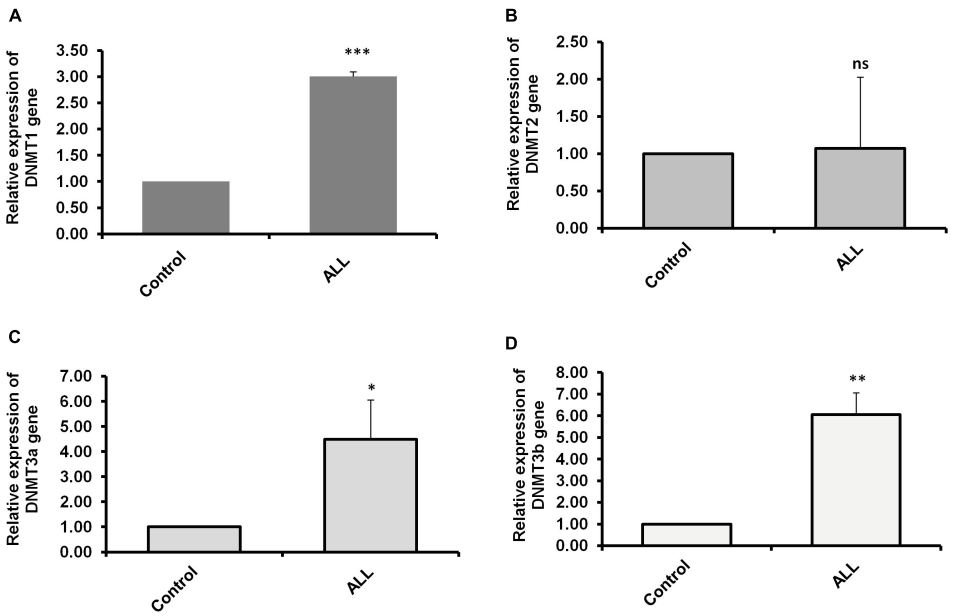

FIGURE 1 | DNMTs expression pattern in collected blood samples from children with ALL. **(A)** The relative gene expression of DNMT1 in obtained samples from children with ALL compared to their expression in healthy individuals indicated by fold change using qRT-PCR. **(B)** The relative gene expression of DNMT2 in samples collected from children with ALL that presented by fold change using qRT-PCR. **(C)** The relative gene expression of DNMT3a in collected samples indicated by fold change using qRT-PCR. **(D)** The relative gene expression of DNMT3b in collected samples indicated by fold change using qRT-PCR. Error bars indicate the standard deviation (SD) of 3 replicates. Student's two-tailed *t*-test was used for statistical analysis of Ct values between patients and healthy individual. (*) indicates *P-values* ≤ 0.05 and (**) indicates the $P \leq 0$.

with ALL, suggesting the activation of DNA methylation in ALL development.

3.3. MS, TET1, and TET2 gene expression is reduced in children with ALL

To further investigate the involvement of DNA methylation in ALL, the relative expression of the TET genes (TET1, TET2, and TET3) and methionine synthase (MS) were monitored in derived samples. Notably, MS expression was significantly downregulated by more than 80% in samples obtained from children with the ALL compared with its expression level in samples derived from healthy children (**Figure 2A** and **Table 7**). In addition, our investigation revealed a marked inhibition of TET1 and TET2 expression in the ALL group when compared to the control samples (**Figures 2B,C** and **Tables 8**, **9**). This finding suggests a decreasing activity of TET enzymes, which are responsible for demethylation processes, and the potential disruptions in the regeneration of methionine, a critical component for DNA methylation, which contributes to DNA methylmoic changes in ALL. Meanwhile, TET3 gene expression showed negligible differences in the ALL patient group compared to the expression of both TET1 and TET2 (**Figure 2D** and **Table 10**), suggesting the impartial role of TET3 in the observed methylomic changes in ALL patients.

3.4. DNA methylation significantly increased within TET1 and TET2 promoter regions in patients with ALL

The converted DNA by sodium bisulfate was amplified with the specific primers targeting the TET1, TET2, and TET3 promoter region sequence to check the methylmoic

TABLE 3 | Meant the average of fold change detected in two replicates of each samples calculated by delta-delta Ct equations and normalized to GAPDH.

Samples	GAPDH		DNMT1		Δ Ct1	Δ Ct2	Δ - Δ Ct1	Δ - Δ Ct2	Ct1 Fold change	Ct2 Fold change	Mean Fold change	STDV	*P*-Value
	MeanCt1	MeanCt2	MeanCt1	Mean Ct2									
Control	18.1	20.91	22.5	23.91	4.4	3.0	0.00	0.00	1.00	1.00	1.0	0.00	
ALL	19.5	20.01	21.7	20.41	2.2	0.4	−1.52	−1.30	2.95	3.06	3.0***	1.55	**0.001**

*** indicates the *p* values ≤ 0.001

TABLE 4 | The mean fold change of DNMT2 expression in obtained samples calculated by delta-delta Ct equations and normalized to GAPDH.

Samples	GAPDH		DNMT2		Δ Ct1	Δ Ct2	Δ - Δ Ct1	Δ - Δ Ct2	Ct1 Fold change	Ct2 Fold change	Mean Fold change	STDV	*P*-Value
	MeanCt1	MeanCt2	MeanCt1	Mean Ct2									
Control	17.9	18.48	26.7	25.76	9.05	7.5	0.00	0.00	1.00	1.00	1.00	0.00	
ALL	16.9	18.28	26.4	25.23	9.68	7.1	0.63	−0.40	0.65	1.49	1.07	0.59	**0.88**

TABLE 5 | The mean fold change of DNMT3a expression in obtained samples calculated by delta-delta Ct equations and normalized to GAPDH.

Samples	GAPDH		DNMT3a		Δ Ct1	Δ Ct2	Δ - Δ Ct1	Δ - Δ Ct2	Ct1 Fold change	Ct2 Fold change	Mean Fold change	STDV	*P*-Value
	MeanCt1	MeanCt2	MeanCt1	Mean Ct2									
Control	17.54	17.01	26.3	25.36	8.24	8.0	0.00	0.00	1.00	1.00	1.00	0.00	
ALL	16.5	17.88	23.3	23.83	6.61	5.7	−2.35	−2.3	3.66	5.32	4.49*	1.17	**0.05**

* indicates the *p* values ≤ 0.05

TABLE 6 | The mean fold change of DNMT3b expression in obtained samples calculated by delta-delta Ct equations and normalized to GAPDH.

Samples	GAPDH		DNMT3b		Δ Ct1	Δ Ct2	Δ - Δ Ct1	Δ - Δ Ct2	Ct1 Fold change	Ct2 Fold change	Mean Fold change	STDV	*P*-Value
	MeanCt1	MeanCt2	MeanCt1	Mean Ct2									
Control	17.9	17.21	26.5	25.56	8.64	8.4	0.00	0.00	1.00	1.00	1.00	0.00	
ALL	16.7	18.08	22.6	24.03	5.93	5.9	−2.71	−2.41	6.78	5.32	6.05**	1.48	**0.01**

** indicates the *p* values ≤ 0.01

alteration within the promoter sequences of the TET gene family. Indeed, bisulfate treatment converts the unmethylated cytosine into uracil in the genomic DNA, while the methylated cytosine is left without alteration. Based on this, the successful amplification of bisulfate converted-DNA by specific primers indicates the level of methylated cytosine and subsequently reveals the methylomic changes in the amplified fragments. Methylation activity within the promoter sequences of the TET gene family was monitored in derived samples using their purified sodium bisulfate converted-DNA and specific oligonucleotides for the TET1, TET2, and TET3 genes. Interestingly, methylation activity within the promoter sequences of the TET1 gene significantly increased in all samples obtained from patients with ALL and reached a 7-fold change when compared with that in control samples (**Figure 3A**). Likewise, methylation activity within the promoter region of the TET2 gene strongly increased up to 20-fold changes in ALL-derived samples compared with that in control-derived samples (**Figure 3B**). Notably, methylation activity within the promoter section of TET3 showed neglected differentiation in samples obtained from patients with ALL compared to that in samples obtained from control group (**Figure 3C**). These findings provide evidence for increasing DNA methylation activity within the promoter region of TET1 and TET2 genes, reducing their expression profile in patients with ALL.

4. Discussions

DNA methylomic change in ALL is modulated by specific DNA methyltransferases (DNMTs) and other regulatory factors like Ten-Eleven Translocation genes (TET). Additionally, the regulation of DNA methylation involves various biochemical markers such as ferritin,

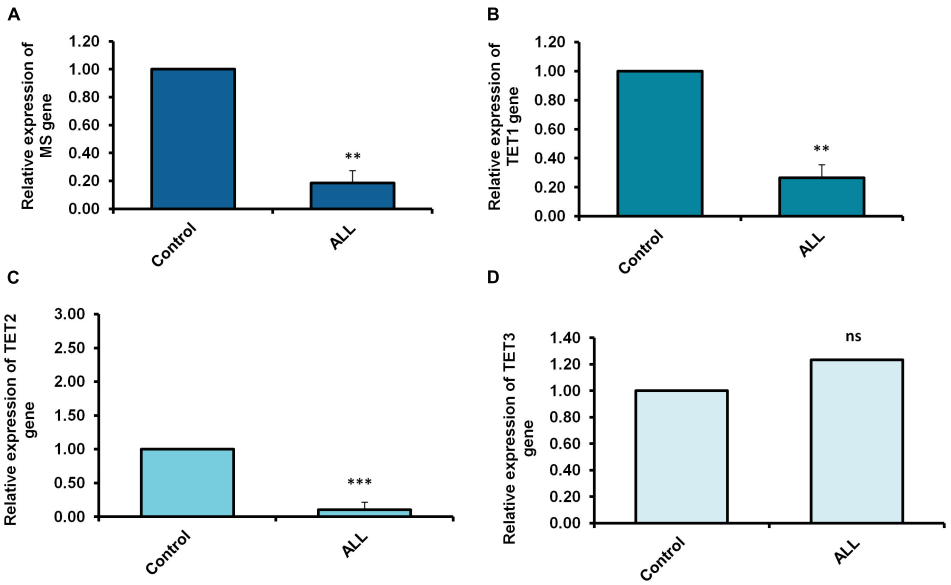

FIGURE 2 | MS and TET expression pattern in samples collected from children with ALL. **(A)** The relative gene expression of MS in obtained samples from children with ALL compared to their expression in healthy individuals indicated by fold change using qRT-PCR. **(B)** The relative gene expression of TET1 in samples collected from children with ALL that presented by fold change using qRT-PCR. **(C)** The relative gene expression of TET2 in collected samples indicated by fold change using qRT-PCR. **(D)** The relative gene expression of TET3 in collected samples indicated by fold change using qRT-PCR. Error bars indicate the standard deviation (SD) of 3 replicates. Student's two-tailed *t*-test was used for statistical analysis of Ct values between patients and healthy individual. (*) indicates *P-values* ≤ 0.05 and (**) indicates the $P \leq 0$.

TABLE 7 | The mean fold change of MS expression in obtained samples calculated by delta-delta Ct equations and normalized to GAPDH.

Samples	GAPDH		MS		Δ Ct1	Δ Ct2	Δ - Δ Ct1	Δ - Δ Ct2	Ct1 Fold change	Ct2 Fold change	Mean Fold change	STDV	*P*- Value
	MeanCt1	MeanCt2	MeanCt1	Mean Ct2									
Control	19.9	19.81	20.31	21.18	0.4	1.37	0.00	0.00	1.00	1.00	1.00	0.00	
ALL	19.5	19.86	19.9	20.15	0.40	0.3	3.37	3.39	0.27	0.10	0.18**	0.17	**0.01**

** indicates the *p* values ≤ 0.01

TABLE 8 | The mean fold change of TET1 expression in obtained samples calculated by delta-delta Ct equations and normalized to GAPDH.

Samples	GAPDH		TET1		Δ Ct1	Δ Ct2	Δ - Δ Ct1	Δ - Δ Ct2	Ct1 Fold change	Ct2 Fold change	Mean Fold change	STDV	*P* Value
	MeanCt1	MeanCt2	MeanCt1	Mean Ct2									
Control	19.9	19.81	20.31	21.18	0.4	1.37	0.00	0.00	1.00	1.00	1.00	0.00	
ALL	20.2	20.53	19.9	20.15	−0.3	−0.4	3.23	2.72	0.37	0.16	0.27**	0.26	**0.010**

** indicates the *p* values ≤ 0.01

TABLE 9 | The mean fold change of TET2 expression in obtained samples calculated by delta-delta Ct equations and normalized to GAPDH.

Samples	GAPDH		TET2		Δ Ct1	Δ Ct2	Δ - Δ Ct1	Δ - Δ Ct2	Ct1 Fold change	Ct2 Fold change	Mean Fold change	STDV	*P*-Value
	MeanCt1	MeanCt2	MeanCt1	Mean Ct2									
Control	17.9	18.81	21.6	20.39	3.66	1.6	0.00	0.00	1.00	1.00	1.00	0.00	
ALL	18.7	19.08	21.2	22.36	2.48	3.3	−0.25	0.27	1.35	1.12	1.23	0.48	**0.1**

vitamin B12, folate, hemoglobin, transferrin saturation, and homocysteine. DNA methylation is a mechanism involved in the epigenetic process that impacts genome stability, transcription, and development and has itsimplication in the pathogenesis and medical conditions, including human malignancies (25). Therefore, it is essential to understand

TABLE 10 | The mean fold change of TET3 expression in obtained samples calculated by delta-delta Ct equations and normalized to GAPDH.

Samples	GAPDH		TET3		Δ Ct1	Δ Ct2	Δ - Δ Ct1	Δ - Δ Ct2	Ct1 Fold change	Ct2 Fold change	Mean Fold change	STDV	*P*-Value
	MeanCt1	MeanCt2	MeanCt1	Mean Ct2									
Control	18.9	19.81	20.3	21.18	1.41	1.4	0.00	0.00	1.00	1.00	1.00	0.00	
ALL	18.5	18.86	24.3	23.72	5.73	4.9	4.32	3.49	0.06	0.15	0.11***	0.10	**0.002**

*** indicates the *p* values ≤ 0.001

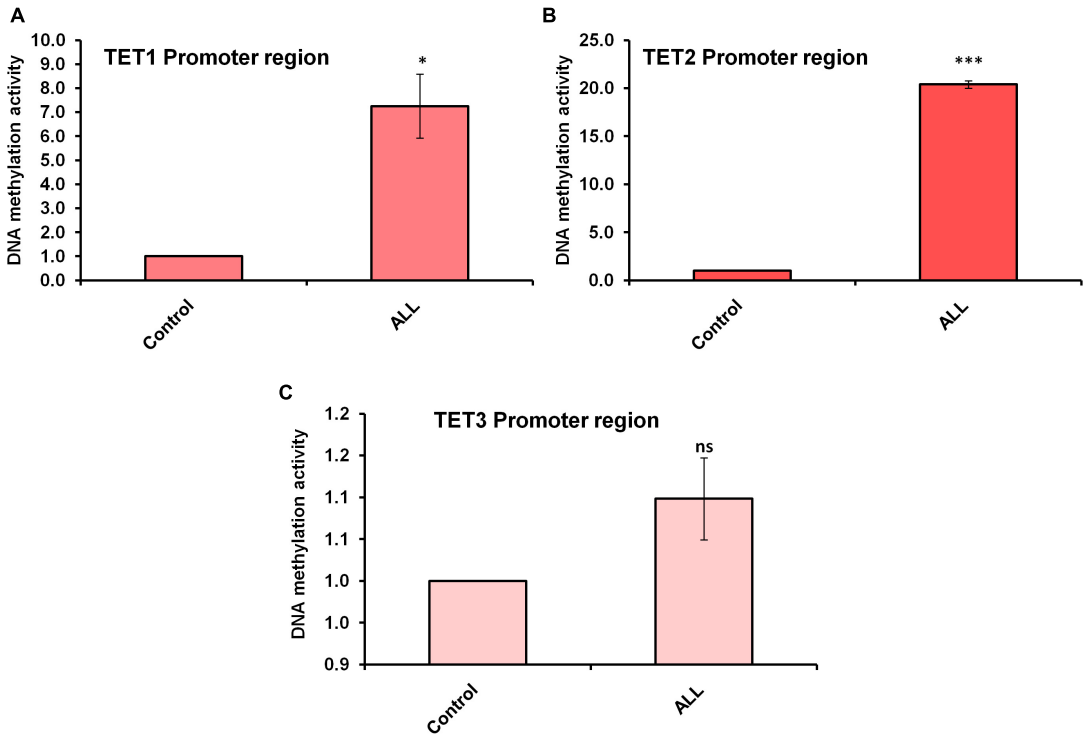

FIGURE 3 | DNA methylation activity in the promoter sections of the TET genes in obtained samples. **(A)** Methylation activity within the promoter region of the TET1 gene in samples obtained from children with ALL compared with its activities in samples obtained from healthy individuals indicated by fold-change using genomic DNA treated with sodium bisulfate. **(B)** Methylation activity within the promoter region of the TET2 gene in patient samples compared with its activities in samples obtained from healthy individuals indicated by fold-change using sodium bisulfate protocol. **(C)** Methylation activity within the promoter region of the TET3 gene in obtained samples from children with ALL compared with its activities in samples obtained from healthy individuals indicated by fold-change using sodium bisulfate protocol. Error bars indicate the SD of 3 different replicates. Student two-tailed *t*-test was used to determine the statistical analysis of Ct values from patients and control groups. (*) indicates *P-values* ≤ 0.05 and (**) indicates the *P* ≤ 0.01.

its role in defining tumor phenotyping. Importantly, in our work, we investigated crucial enzymes that regulate the DNA methylation progress, such as DNMTs and TETs, which have been described as being altered in their expression in ALL cancer with severe consequences. They also regulate the dynamics between hyper and hypomethylation (26). Numerous studies have suggested the clear connection of DNA methylation activity and the levels of some biochemical markers, including vitamin B12, ferritin, homocysteine, and saturated transferrin in ALL. These markers may have relevance in other biological processes or metabolic pathways that mediate the DNA methylation pathway and affect DNA methylation aberrations. Intriguingly, our results showed that the number of patients in the ALL group who were deficient in vitamin B12, folate, hemoglobin, transferrin saturation, and homocysteine was significantly higher than that in the control group. These findings suggest a potential link between these nutrient deficiencies, the DNA hypermethylation process, and the gene silencing of TET1 and TET2. Interestingly, our findings showed that DNMT1, DNMT3a, and DNMT3b were overexpressed in the ALL children with depleted levels of vitamin B12, folate, and ferritin, compared to the healthy children group. This finding supports the concept suggesting that vitamin B12 and folate play a role in methylomic changes of genomic DNA during ALL progression. For further interpretation, our data also showed a substantial depletion in the MS expression pattern in all children with low vitamin B12 and ferritin levels. Importantly, other studies have reported the association between vitamin B12 and methylomic

changes in genomic DNA due to its recognized role in the metabolism of methionine (8, 27, 28). Consequently, diminished MS protein activity reduces methionine levels, a significant cofactor of DNA (cytosine-5)-methyltransferase 1. This protein regulates methionine levels because it is a precursor for SAMe metabolism that donates methyl groups necessary for cellular DNA methylation (29). To further understand how DNA hypermethylation of particular genes could have occurred during the development of acute lymphocytic leukemia, we focused on TET enzymes, including TET1, TET2, and TET3, which are the most crucial demethylating tools that determine methylation levels and tumor suppression (30). Here, we studied the relative expression of TET genes in ALL and observed the significant downregulation of TET1 and TET2 in samples obtained from children with ALL compared with healthy individual groups. The decrease in TET levels affects the demethylation process, which leads to various physiological conditions such as hematological malignancies AML, ALL, myelodysplastic syndromes (MDS), and chronic myelocytic leukemia (CML) (31). Considering the observation that TET1 and TET2 oxidize 5-methylcytosines promote demethylation activity of DNA (32), we postulate that the downregulation of TET1 and TET2 gene expression may contribute to the DNA hypermethylation of malignant cells. This downregulation of TET1 and TET2 expression is likely influenced by epigenetic alterations in their promoter sections, which influence their expression profile in ALL (33). Furthermore, several studies have suggested that TET methylcytosine dioxygenase proteins help to stop the development and transformation of cancer (32, 34). TET generates 5-hmC by oxidizing 5-mC in an iron- and alpha-ketoglutarate-dependent mechanism (13). The conversion of 5-mC into 5-hmC is considered as the initial step in the active DNA demethylation process (35). Other evidence indicates that abnormal expression of members of the TET family has been found in a wide range of human malignancies, such as solid tumors and hematological cancers, in AML and ALL (32). TET1 has been reported to play dual roles in hematological malignancies depending on the cellular lineage, whereas, TET 1 can be transcriptionally inactivated, leading to a block in 5 mC-to-5 hmC conversion and, thus, DNA demethylation (36).

The abnormal expression of transcriptional effectors incorporates in B-cell ancestry condition, chromosome preservation, and DNA repair. Depleted TET1prtein increases the self-regeneration of lymphocyte cells and cooperates with Bcl2 overexpression, leading to an abnormal increase in B lymphocytes. TET1-depleted pro-B cells also exhibited increased DNA damage. These findings provide valuable evidence supporting the role of TET1 as a tumor suppressor in hematopoietic malignancies (37). However, the oncogenic role of the TET2 mutations has been observed in various hematological disorders, including both myeloid and lymphoid cancers (3, 13). It is worth mentioning that the TET2 mutations have been observed in conjunction with DNMT3a mutations, implying a potential co-evolution of both mutations during the development of malignancy (38). This finding supports the assumption that patients with TET2 mutations had a hypermethylation signature (39).

5. Conclusion

We analyzed the methylomic changes associated with nutrient levels in children with ALL compared with healthy children by measuring the relative gene expression of epigenetics-related factors. Interestingly, the analyzed biochemical factors indicated the correlation between the levels of ferritin, vitamin B12, and serum folate and the expression pattern of epigenetic-related factors. The quantification of gene expression in derived samples demonstrated that DNMT1, DNMT3a, and DNMT3b gene expression displayed strong upregulation in children with ALL. Alternatively, MS, TET1, and TET2 gene expression revealed a noticeable decrease in children with ALL in parallel with deficient levels of ferritin, vitamin B12, serum folate, hemoglobin, transferrin saturation, and homocysteine, which exhibits the correlation between them and methylomic changes in ALL.

Author contributions

RR and DR carried out the experiments. AG assisted in supervising and conceptualizing experiments. HK designed the research plan, led the overall research, provided and interpreted data. RR and HK wrote the manuscript. All authors contributed to the article and approved the submitted version.

References

1. Shafique S, Tehsin S. Acute lymphoblastic leukemia detection and classification of its subtypes using pre-trained deep convolutional neural networks. *Technol Cancer Res Treat.* (2018) 17:1533033818802789.
2. Marincevic-Zuniga Y. *Resolving the genomic complexity of pediatric acute lymphoblastic leukemia. Digital comprehensive summaries of Uppsala dissertations from the faculty of medicine 1452*. Sweden: Uppsala University (2018).
3. Scourzic L, Couronné L, Pedersen M, Della Valle V, Diop M, Mylonas E, et al. DNMT3A R882H mutant and Tet2 inactivation cooperate in the deregulation of DNA methylation control to induce lymphoid malignancies in mice. *Leukemia.* (2016) 30:1388–98.
4. An J, Ko M. Epigenetic Modification of Cytosines in Hematopoietic Differentiation and Malignant Transformation. *Int J Mol Sci.* (2023) 24:1727.
5. Guirgis S, El-Halfawy K, Alalem M, Khalil H. Legionellapneumophila induces methylomic changes in ten-eleven translocation to ensure bacterial reproduction in human lung epithelial cells. *J Med Microbiol.* (2023) 72:1676.

6. Ehrlich M. DNA hypermethylation in disease: mechanisms and clinical relevance. *Epigenetics.* (2019) 14:1141–63.
7. Boughanem H, Hernandez-Alonso P, Tinahones A, Babio N, Salas-Salvadó J, Tinahones F, et al. Association between serum vitamin B12 and global dna methylation in colorectal cancer patients. *Nutrients.* (2020) 12:1–12.
8. Ge Y, Zadeh M, Mohamadzadeh M. Vitamin B12 regulates the transcriptional, metabolic, and epigenetic programing in human ILEAL epithelial cells. *Nutrients.* (2022) 14:2825.
9. Li H, Chiappinelli K, Guzzetta A, Easwaran H, Chiu Yen R, Vatapalli R, et al. Immune regulation by low doses of the DNA methyltransferase inhibitor 5-azacitidine in common human epithelial cancers [Internet]. *Oncotarget.* (2014) 5:587–98.
10. Kok D, Dhonukshe-Rutten R, Lute C, Heil S, Uitterlinden A, Van Der Velde N, et al. The effects of long-term daily folic acid and vitamin B12 supplementation on genomewide DNA methylation in elderly subjects. *Clin Epigenetics.* (2015) 7:1.
11. Baylin S, Jones PA. A decade of exploring the cancer epigenome-biological and translational implications. *Nat Rev Cancer.* (2011) 11:726–34.
12. Tahiliani M, Koh K, Shen Y, Pastor W, Bandukwala H, Brudno Y, et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science.* (2009) 324:930–5.
13. Koh K, Yabuuchi A, Rao S, Huang Y, Cunniff K, Nardone J, et al. Tet1 and Tet2 regulate 5-hydroxymethylcytosine production and cell lineage specification in mouse embryonic stem cells. *Cell Stem Cell.* (2011) 8:200–13.
14. Shekhawat J, Gauba K, Gupta S, Choudhury B, Purohit P, Sharma P, et al. Ten–eleven translocase: key regulator of the methylation landscape in cancer. *J Cancer Res Clin Oncol.* (2021) 147:1869–79.
15. Salminen A, Kauppinen A, Kaarniranta K. 2-Oxoglutarate-dependent dioxygenases are sensors of energy metabolism, oxygen availability, and iron homeostasis: Potential role in the regulation of aging process. *Cell Mol Life Sci.* (2015) 72:3897–914.
16. Wang Y, Yu L, Ding J, Chen Y. Iron metabolism in cancer. *Int J Mol Sci.* (2019) 20:95.
17. Brown R, Richardson K, Kabir T, Trinder D, Ganss R, Leedman P. Altered iron metabolism and impact in cancer biology, metastasis, and immunology. *Front Oncol.* (2020) 10:476. doi: 10.3389/fonc.2020.00476
18. Biamonte F, Battaglia A, Zolea F, Oliveira D, Aversa I, Santamaria G, et al. Ferritin heavy subunit enhances apoptosis of non-small cell lung cancer cells through modulation of miR-125b/p53 axis. *Cell Death Dis.* (2018) 9:1174.
19. Akcam M, Ozdem S, Yilmaz A, Gultekin M, Artan R. Serum Ferritin, Vitamin B12, Folate, and Zinc Levels in Children Infected with *Helicobacter* pylori. *Dig Dis Sci.* (2007) 52:405–10.
20. Elawdan K, Farouk S, Aref S, Shoaib H, El-Razik M, Abbas N, et al. Association of vitamin B12/ferritin deficiency in cancer patients with methylomic changes at promotors of TET methylcytosine dioxygenases. *Biomark Med.* (2022) 16:959–70.
21. Luo G, Wang F, Weng X, Chen K, Hao Z, Yu M, et al. Characterization of eukaryotic DNA N 6 -methyladenine by a highly sensitive restriction enzyme-assisted sequencing. *Nat Commun.* (2016) 7:11301.
22. Abd El Maksoud A, Elebeedy D, Abass N, Awad A, Nasr G, Roshdy T, et al. Methylomic Changes of Autophagy-Related Genes by Legionella Effector Lpg2936 in Infected Macrophages. *Front Cell Dev Biol.* (2020) 7:390. doi: 10.3389/fcell.2019.00390
23. Alalem M, Dabous E, Awad A, Alalem N, Guirgis A, El-Masry S, et al. Influenza a virus regulates interferon signaling and its associated genes; MxA and STAT3 by cellular miR-141 to ensure viral replication. *Virol J.* (2023) 20:183.
24. El-Fadl H, Hagag N, El-Shafei R, Khayri M, El-Gedawy G, Maksoud A, et al. Effective Targeting of Raf-1 and Its Associated Autophagy by Novel Extracted Peptide for Treating Breast Cancer Cells. *Front Oncol.* (2021) 11:682596. doi: 10.3389/fonc.2021.682596
25. Ehrlich M. DNA hypomethylation in cancer cells. *Epigenomics.* (2009) 1:239–59.
26. Ghavifekr Fakhr M, Farshdousti Hagh M, Shanehbandi D, Baradaran B. DNA Methylation Pattern as Important Epigenetic Criterion in Cancer. *Genet Res Int.* (2013) 2013:1–9.
27. Halczuk K, Kaźmierczak-Barańska J, Karwowski B, Karmańska A, Cieślak M. Vitamin B12—Multifaceted In Vivo Functions and In Vitro Applications. *Nutrients.* (2023) 15:2734.
28. Konda M, Godbole A, Pandey S, Sasapu A. Vitamin B12 deficiency mimicking acute leukemia. *Baylor Univ Med Center Proc.* (2019) 32:589–92.
29. Bordbar M, Haghpanah S, Shakibazad N, Zarei T. Serum Folate and Vitamin B12 Levels in Survivors of Childhood Malignancy in Southern Iran: 202-207. *Middle East J Cancer.* (2018) 9:202–7.
30. Jones P, Baylin S. The epigenomics of cancer. *Cell.* (2007) 128:683–92.
31. Liu W, Wu G, Xiong F, Chen Y. Advances in the DNA methylation hydroxylase TET1. *Biomarker Res.* (2021) 9:76.
32. Rasmussen K, Helin K. Role of TET enzymes in DNA methylation, development, and cancer. *Genes Dev.* (2016) 30:733–50. doi: 10.1101/gad.276568.115
33. Suzuki T, Komatsu T, Shibata H, Tanioka A, Vargas D, Kawabata-Iwakawa R, et al. Crucial role of iron in epigenetic rewriting during adipocyte differentiation mediated by JMJD1A and TET2 activity. *Nucleic Acids Res* (2023) 51:6120–42.
34. Scourzic L, Mouly E, Bernard OA. TET proteins and the control of cytosine demethylation in cancer. *Genome Med.* (2015) 7:9.
35. Lio C, Rao A. TET enzymes and 5hMC in adaptive and innate immune systems. *Front Immunol.* (2019) 10:210. doi: 10.3389/fimmu.2019.00210
36. Jiang H, Ou Z, He Y, Yu M, Wu S, Li G, et al. DNA methylation markers in the diagnosis and prognosis of common leukemias. *Signal Transduct Target Ther.* (2020) 5:3.
37. Hsu C, Peng K, Kang M, Chen Y, Yang Y, Tsai C, et al. TET1 Suppresses Cancer Invasion by Activating the Tissue Inhibitors of Metalloproteinases. *Cell Rep.* (2012) 2:568–79.
38. Ito S, Shen L, Dai Q, Wu S, Collins L, Swenberg J, et al. Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science.* (2011) 333:1300–3.
39. Fakultät D, Ravichandran M. *Biochemical Characterisation of TET DNA Hydroxylases*. Stuttgart: University of Stuttgart (2017).