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Global DNA Methylation Levels in Epstein-Barr-Virus-Positive Iraqi Patients with Acute Lymphoblastic Leukaemia

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Abstract

Acute lymphoblastic leukemia (ALL) is one of the commonest hematological malignancies affecting children and adults. Recent evidence suggests an involvement of Epstein-Barr virus (EBV) in ALL pathogenicity. Epigenetic aberration, especially altered DNA methylation marks, is a key event of cancer development. The present study aims to investigate how the ALL epimethylome reacts to viral infection through the assessment of the total 5-methylcytosine (5mC) levels in ALL patients, according to EBV infection. The 5mC global DNA methylation levels in 50 diagnosed ALL patients (age mean 26.23 yrs; age range 10-60 yrs) and 25 age-matched healthy controls were assessed using MethylFlash™ Methylated DNA Quantification Kit. Acute primary EBV infection in the studied subjects was detected by measuring Epstein-Barr Virus (EBNA-1) IgG levels using ELISA.

The results showed a significant ($P<0.001$) decrease in 5mC levels in ALL-EBV positive cases as compared to those who were negative for EBV infection (0.234±0.117 vs. 0.441±0.153, respectively). The reduction in the average 5mC level seemed to be negatively correlated with EBV viral load ($r = -0.599$, $p= 0.001$). Additionally, 5mC levels were able to distinguish between ALL main subtypes (B-ALL and T-ALL; derived from B or T lymphocytes), where T-ALL cases showed significantly ($P=0.005$) higher 5mC levels than those of B-ALL cases (0.587±0.070 vs. 0.180±0.092, respectively). Also significantly ($P=0.04$) lower 5mC levels were detected in Philadelphia chromosome-positive (Ph^+) ALL cases than in those who were negative to this genetic abnormality (Ph^- -ALL) (0.13±0.021 vs. 0.179±0.093, respectively). Overall, the findings of the present study suggest an involvement of EBV infection in ALL pathogenicity, with the potential of utilizing the differences in global DNA methylation levels in ALL patients' risk stratification.

Key words: DNA methylation, Acute lymphoblastic leukemia, EBV

مستويات مثيلة الحمض النووي الكلية في المرضى العراقيين المصابين بسرطان الدم الليمفاوي الحاد الايجابي لفيروس ابشتاين بار

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ابيضاض الدم الليمفاوي الحاد Acute lymphoblastic leukemia (ALL) هو أحد أكثر الأورام الدموية الخبيثة شيوعًا التي تصيب الأطفال والبالغين. تشير الدراسات الحديثة إلى دور محتمل لفيروس إبشتاين بار (EBV) في امراضية الـ ALL. تعد الانحرافات اللاجينية، وخاصة تغايرات مثيلة الحمض النووي، حدثًا بيولوجيا مهما في نشوء السرطان وتطوره .تهدف الدراسة الحالية إلى بيان التأثير المحتمل للإصابة بفايروس إبشتاين بار EBV على الأبيجينوم لمرضى ابيضاض الدم الليمفاوي الحاد من خلال تقييم مستويات مثيلة الدنا الكلية 5mC في المرضى وفقًا لعدوى EBV. تم تقييم مستويات مثيلة الحمض النووي الكلية 5mC في 50 مريضًا (متوسط العمر 26.23 عامًا ؛ الفئة العمرية 10–60 عامًا) و 25 فردا من الأصحاء (مجموعة السيطرة) باستخدام MethylFlash ™ Methylated DNA Quantification Kit. وتم الكشف عن عدوى EBV الأولية الحادة في الحالات المدروسة عن طريق قياس مستويات IgG لمستضدات فيروس ابشتاين النووية EBNA–1 باستخدام تقنية الأليزاELISA.

أظهرت النتائج انخفاضًا معنويًا ($P<0.001$) في مستويات 5mC في الحالات الإيجابية لـ EBV–ALL مقارنةً بتلك التي كانت سلبية لعدوىEBV (0.117 ± 0.234 مقابل 0.441 ± 0.153 ، على التوالي). وبينت النتائج أن الانخفاض في متوسط مستوى 5mCمرتبط عكسيا مع شدة الاصابة الفيروسية EBV viral load ($r = -0.599$ ، $P = 0.001$). بالإضافة إلى ذلك ، كانت مستويات 5mC قادرة على التمييز بين جميع الأنواع الفرعية لمرض ALL (B–ALL و T–ALL ؛ مشتق من الخلايا الليمفاوية B أو T) ، حيث أظهرت حالات T–ALL مستويات 5mC أعلى من تلك الموجودة في حالات B–ALL (0.587 ± 0.070 مقابل 0.180 ± 0.092 على التوالي$P = 0.005$). أيضًا أظهرت النتائج مستويات أقل من 5mC في حالات المرض الإيجابية لكروموسوم فيلادلفيا (Ph + ALL) مقارنة بالمرضى الذين لا يحملون هذا الأضطراب الجيني(0.021 ± 0.13 مقابل 0.179 ± 0.093، على التوالي) . بشكل عام ، تشير نتائج الدراسة الحالية إلى اهمية عدوى EBV في امراضية ابيضاض الدم الليمفاوي الحاد، مع إمكانية الاستفادة من الاختلافات في مستويات مثيلة الحمض النووي الكلية في تصنيف المرضى الى مجاميع ثانوية اعتمادا على عوامل الخطورة للمرض patients' risk stratification.

1. Introduction

DNA methylation is an epigenetic modification that involves the addition of methyl group to the cytosine in the CpG site. This reaction is catalyzed by DNA methyletransferases (DNMTs) enzyme family. The total methyl attached to the DNA CpG sites is referred to as global DNA methylation [1]. Acute lymphoblastic leukemia (ALL) is one of the most common hematological malignancies, with 80 % of cases occurring in children while 20 % are manifested in adults. ALL prognosis is much worse in adults than in children, with approximately 30-40% of adults with ALL achieving 5 years overall survival in comparison to up to 80-90% in children [2, 3]. In respect to ALL causality, several lines of evidence have suggested that a combination of genetic and epigenetic alterations is accountable for ALL development [4-6]. Within this context, epigenetic alterations that exist in all human malignancies are believed to precede cellular transformation-associated genetic events [7]. Epigenetic alterations, including DNA methylation and histone modifications, are thought to constitute the precancerous transformation events that proceed the known cancer-associated genetic abnormalities [8, 9]. Considering that both the integrity and the transcription activity of the genome are maintained and governed by the epigenome, cancer-associated genomic instability has been linked to the disrupted epigenetic marks. This highlights the importance of interrogating cancer-specific epigenetic marks for the development of disease-related biomarkers, especially those with the potential use in patients' stratification [10, 11].

Furthermore, it is generally acknowledged that cancer initiation and development, and ALL not being an exception, are caused by a combination of risk factors, such as the interaction of genetic and environmental factors (including oncogenic viruses). It is also suggested that oncogenic viruses encode certain epigenetic factors that lead to the immortality

and proliferation of infected cells [12, 13]. Abnormal immune responses to infections have been suggested to be implicated in the childhood leukaemia development [14]. Increasing evidence has revealed that oncogenic viruses contribute to the epigenetic changes that are considered hallmarks of cancer initiation and progression. Cancer-associated viruses can interfere with the epigenetic machinery and confer aberrations of DNA methylation as well as changes in histone modifications affecting the transpiration profile of the host cell [15]. Previous studies have implicated Epstein-Barr virus (EBV) in the pathogenesis of a number of serious illnesses, including autoimmune diseases, chronic lymphocytic leukaemia (CLL) and other lymphocyte disorders. EBV is one of the most common human viruses worldwide. It infects B lymphocytes via binding to their CD21surface protein receptor [16]. Recent findings suggest the involvement of EBV in ALL, based on the EBNA3 enrichment , a transcription factor encoded by the EBV genome, in ALL genome [17, 18]. In addition, studies have shown that EBV^{+}-ALLs had higher relapse and mortality rates than those of EBV^{-}-ALLs [19].

Since the epigenome is more vulnerable than the genome to environmental insults, including viral infections such as EBV, it would be interesting to assess the global DNA methylation level in ALL patients, especially those who are EBV positive. This would provide useful information to understand how the ALL epimethylome reacts to EBV infection, with the hope of utilizing such knowledge in the disease's management and prevention strategies.

2. Subjects, Materials and Methods

Blood samples (5ml) were collected from the participants (50 ALL patients and 25 healthy controls). ALL patients (25 females and 25 males) were diagnosed at blood diseases center-Baghdad medical city, Baghdad, Iraq during the period of October 2020 to May 2021. The age average of the participants was 26.23 years (ranging from 10-60 yrs.). This study was approved by the Ethical Committee, Department of Biology, College of Science, University of Bagdad and the Iraqi Ministry of Health, Baghdad, Iraq**.**

2.1. Detection of Anti-EBV IgG by Enzyme Linked Immunosorbent Assay (ELISA)

The detection of acute primary Epstein-Barr Virus (EBV) infection is principally serology-based, as previously described [20]. This was accomplished by using Epstein-Barr Virus (EBNA-1) IgG ELISA-kit (Demeditec Diagnostics GmbH, Germany) following the manufacturer's instructions.

2.2. DNA Extraction and Global DNA Methylation Assessment

Genomic DNA was extracted from the studied ALL cases and healthy controls using ReliaPrep™ Blood gDNA Miniprep System, Promega, USA. The concentrations and the extracted DNA purity were then quantified by NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). In respect to the estimation of global DNA methylation levels in the extracted DNA samples, MethylFlash™ Global DNA Methylation (5 mC) ELISA Easy Kit (Catalog # P-1034, Epigentek, USA) was utilized. This was performed by diluting 100ng of the extracted genomic DNA from each tested sample in the supplied binding solution provided with an eight-well-assay strips kit. In principle, the DNA methylation fraction that binds to the well-assay strips monoclonal antibodies is captured to be detected by the subsequent assay steps. These included the addition of wash solution, detection antibody, enhancer solution, developer and stop reaction solution. Ultimately, global DNA methylation quantification was calculated as proportional to the OD intensity read (at 450nm) using micro-plate reader (Thermo Fisher Scientific Inc.), based on the manufacturer instructions.

Global DNA methylation perecentage was proportionally measured by subtracting the OD of the positive controls, supplied by the kit, from the OD of each tested sample. To ensure obtaining reliable generated signals, all of the analyzed samples were run in duplicate, along with the use of positive and negative controls provided by the kit.

2.3. Statistical Analysis

The obtained results were then tabulated and analyzed using Microsoft Excel version 2013. The mean values of the investigated parameters were compared among the studied groups by using t-Test. In addition, the correlation regression analysis (Pearson correlation coefficient) was performed online by using http://vassarstats.net/ website.

3. Results and Discussion

3.1. Global DNA Methylation Levels Significantly Reduced in ALL-EBV Positive Cases

Our results showed significantly (P<0.001) lower levels of 5mC in ALL-EBV positive cases than those who were negative for EBV infection (0.234±0.117 vs. 0.441±0.153, respectively, Figure 1). The average of global DNA methylation (5mC %) was reduced by nearly half (47%) in ALL-EBV+ cases than that of ALL-EBV- cases. Our study finding is in consistence with that of Dhabi *et al.* who found out that the gene promoters hypermethylation were more frequently detected in EBV-negative than in EBV-positive Hodgkin lymphomas cases [21]. This implicates the impact of EBV infection in the modulation of leukemic epimethylome through increasing the global DNA hypomethylation, and the latter change is generally acknowledged to be a key event in cancer development [22].

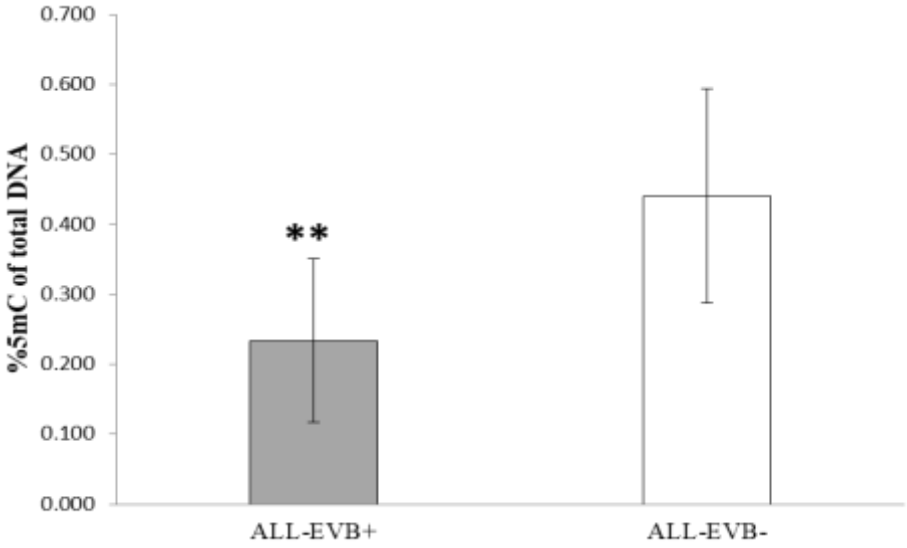

Figure 1: The average levels of 5mC in ALL cases according to EBV infection. Data are presented as mean ± standard deviation (SD). **P<0.01.

3.2. The Higher EBV Load Contributes to the Lower Global DNA Methylation in the Assessed ALL Cases

The results showed that the higher EBV load corresponds to lower 5mC levels in the studied ALL cases. The reduction in the average level of 5mC was just above 50% in ALL patients with higher EBV load than those with lower viral load (0.195±0.097 vs. 0.409±0.140, respectively, Figure 2). This increase in global DNA hypomethylation resulted in significant difference (P= <0.001) between ALL patients with higher and lower EBV load. Pearson correlation coefficient was computed to assess the relationship between EBV viral load and 5mC. Of interest, there was a strong negative correlation between the two assessed variables

(EBV viral load and total 5mC; r = -0.599, p= 0.001). In line with this finding, other studies have also shown a global DNA hypomethylation profile of B cells experimentally infected with EBV [23].

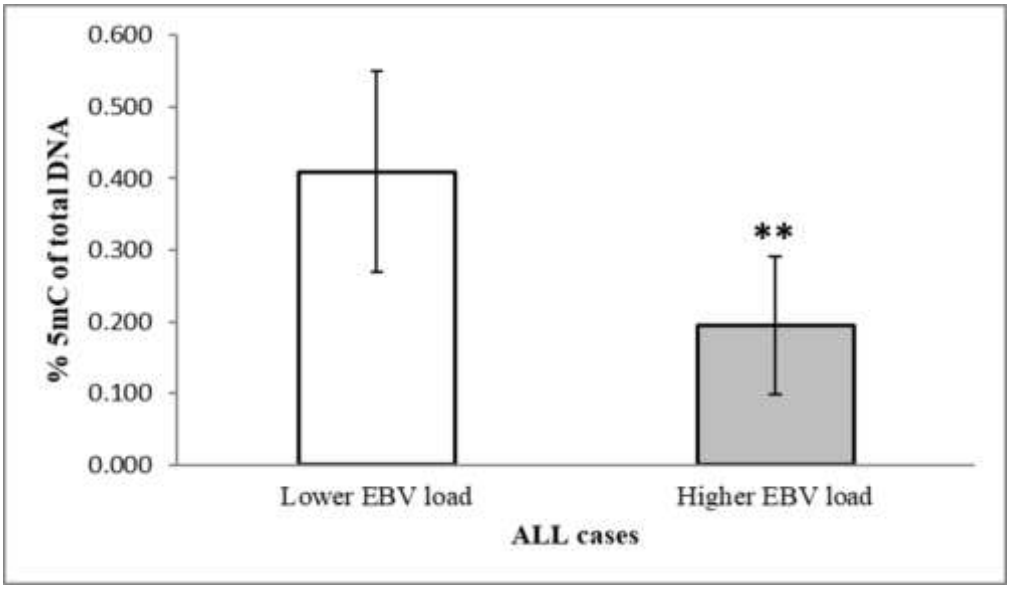

Figure 2: The levels of 5mC in the total DNA of ALL patients according to the EBV load. Data is presented as mean ± standard deviation (SD). **P<0.01.

3.3. Global DNA Methylation Levels Allow the Discrimination between Different ALL Main Subtypes

Further support to the notion of the contribution of epigenetic aberrations, especially global DNA hypomentylation, in cellular transformation was obtained from the comparison of the 5mC levels in ALL cases and their age-matched healthy controls. ALL cases showed significantly lower average 5mC levels (0.413±0.158 vs. 0.248±0.125, respectively, Figure 3). The global DNA methylation levels in ALL patients were reduced by about 40% than those of their healthy counterparts. This seems to be consistent with the large body of evidence that show global DNA hypomethylation in malignant tissues in comparison to the healthy counterpart [24, 25].

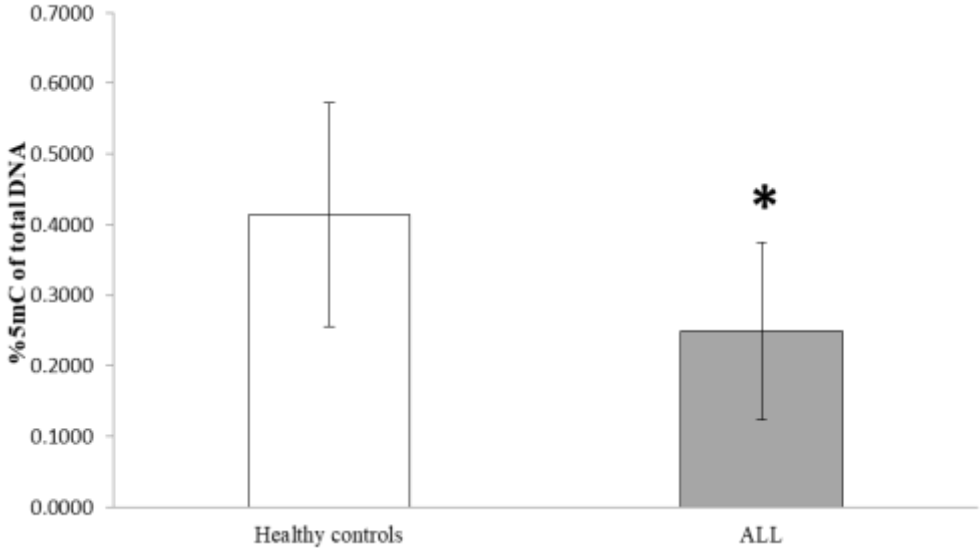

Figure 3: Global DNA methylation levels (%5mC of total DNA) in ALL cases and the healthy control group. Data are presented as mean ± standard deviation (SD). *P<0.05.

Interestingly, the 5mC levels were able to distinguish between ALL main subtypes (B-ALL and T-ALL, derived from B or T lymphocytes). T-ALL cases showed significantly (P=0.005) higher 5mC levels than those of B-ALL cases (0.587±0.070 vs. 0.180±0.092, respectively, Figure 4). In the investigated set of leukaemia patients, the global DNA methylation of B-ALL was found to have been decreased by about 70% than that of T-ALL cases.

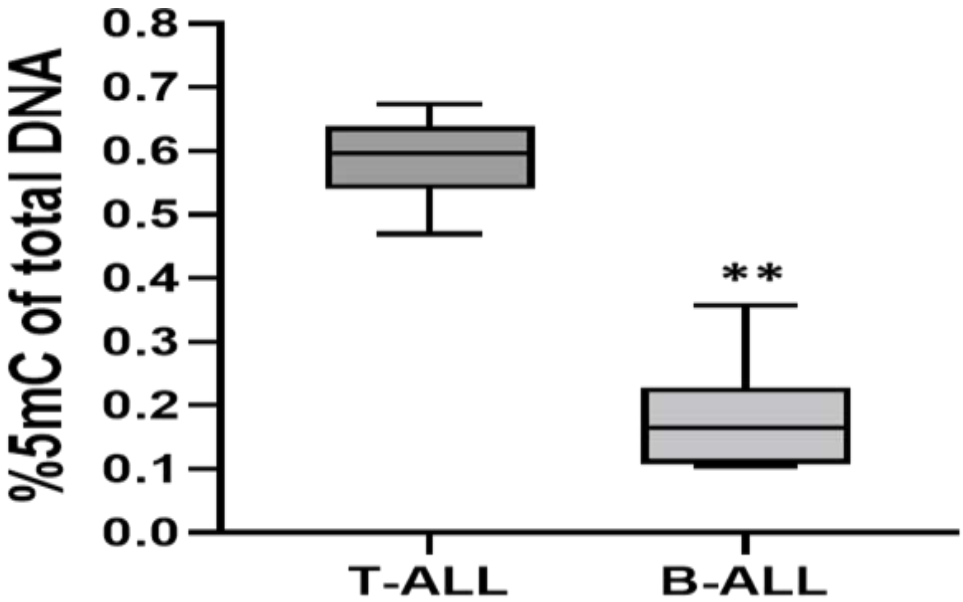

Figure 4: 5mC levels in the assessed ALL main subtypes (B-ALL and T-ALL). Data are presented as mean ± standard deviation (SD). **P<0.01.

Another interesting observation in the present study is the lower 5mC levels in Philadelphia chromosome-positive (Ph^+) ALL cases than in those who were negative to this cytogenetic abnormality (Ph^- -ALL) (0.179±0.093 vs.0.13±0.021, respectively, Figure 5). The 5mC of the total DNA was significantly (P=0.04) lower by 27.37% in the Ph negative ALL patients in comparison to that of Ph positive ALL cases. These subtype-specific patterns of DNA methylation might contribute to the differential outcome seen in the different ALL subtypes as stated by a number of recent studies [11, 26, 27].

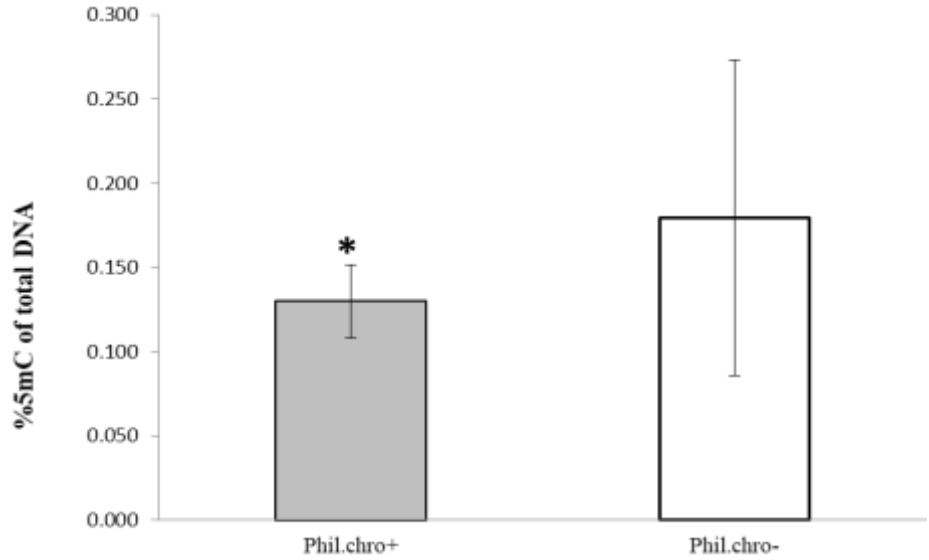

Figure 5: Total 5mC of DNA levels in the studied ALL cases according to Philadelphia chromosome status. Data are presented as mean ± standard deviation (SD). *P<0.05.

A plethora of evidence has been suggesting the involvement of epigenetic alterations, especially aberrations in DNA methylation, in cancer initiation and progression [28-31]. Viral infections account for the etiology of more than 15% of human cancers [32]. Oncoviruses are believed to cause epigenetic changes which play a crucial role in carcinogenesis. Viral oncoproteins prompt transformed cells to evade growth suppression and apoptosis, sustain proliferation and induce genome instability [33].

Our study findings showed that the 5mC of the total DNA significantly reduced in ALL patients who were EBV positive in comparison to their uninfected counterparts. In addition, the levels of 5mC were shown to be reduced by 40% in ALL patients compared to those in the healthy controls. Of interest, this results seemed to agree with that of Hansen and colleagues who reported large-scale hypomethylated blocks comprising two-thirds of the B-cell genome induced by EBV immortalization [34]. These finding suggest the impact of EBV viral infection in ALL pathogenicity which ought to be investigated further for its utility in the development of disease detection and prevention strategies. Recently, the role of EBV infection in the pathogenesis of ALL has been explained by a mechanism operating in transformed B cells through the EBV latency III program of viral expression [35]. However, what is not clear yet is the impact of EBV infection on the ALL epimethylome. Considering the long incubation period of oncoviruses, the occurrence of virus-induced cancers may take several years to manifest following the initial viral infection [36, 37]. However, distinguishing oncovirus-associated DNA methylation changes from those linked to the host antiviral response is one of the challenges [22]. It is suggested that EBV infection induces alterations in N6-methyladenosine (m^6A) RNA and acts as transcription regulator of the methylation of viral and host cell mRNA. These epitransciptomic changes were shown to promote EBV infection *in vitro* [38, 39]. Furthermore, accumulating evidence demonstrated that abnormal changes in the m^6A levels are associated with human tumorigenesis and drug resistance [40-42].

Interestingly the finding of our study showed that the 5mC levels were able to distinguish between ALL main subtypes of B-ALL and T-ALL, in addition to the significant differences in the 5mC levels between ALL patients with different prognostic potential (e.g. Ph^+ vs. Ph^- cases). Indeed, the utility of epigenetic landscape has been recently suggested for cancer patient's stratification, especially those misclassified. Moreover, genetically unclassified childhood could be categorized into specific cytogenetic subgroups, based on their methylome similarity to the known genetic subtypes [43, 44]. In respect to the association of 5mC with the cytogenetic abnormality of Philadelphia chromosome, large scale studies are recommended to investigate the prognostic potential of this association in relation to DNA methylation changes associated with EBV infection in ALL patients.

4. Conclusions

Overall, the findings of the present study suggest a possible involvement of EBV infection in ALL pathogenicity, with the potential of utilizing the levels of global DNA methylation for the risk stratification of ALL patients.

"Conflict of Interest: The authors declare that they have no conflicts of interest."
"All experiments were conducted in accordance with Helsinki Declaration of 1975, as revised in 2000. Informed consent for all human subjects included in the study was also obtained.

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