Abstract

Background: Investigation of DNA methylation in Alu repetitive elements (REs) was shown to be a promising field to explore transcriptional changes in human genome under disease condition. To scrutinize the association between Alu methylation and tuberculosis (TB) disease in children, the difference in Alu DNA methylation level was compared with healthy controls. Methods: Whole-blood genomic DNA from 36 TB-infected children and 32 healthy controls was isolated, and the level of Alu repeat DNA methylation was examined by methylation-specific polymerase chain reaction. Results: The median Alu methylation level in TB patients was 30% (Interquartile range [IQR], 25–30%), whereas in healthy controls, it was 75% (IQR, 50–75%) (P < 0.0001). The median level of DNA methylation of Alu RE in TB cases was significantly lower than healthy controls. Receiver operating characteristic curve analysis showed that the area under the curve for diagnosis was 0.969 (95% confidence interval, 0.936–1) (P < 0.0001), with 100% sensitivity and 84% specificity. Conclusion: Our results point out that detection of Alu DNA methylation in whole-blood DNA may be clinically useful tool for the diagnosis and prognosis of TB disease in children.

Keywords: Alu repeats, DNA methylation, hypomethylation, methylation-specific polymerase chain reaction, tuberculosis

INTRODUCTION

One-third of the world population is infected with tuberculosis (TB) and approximately 10% people develop into active disease.[1] Around 2.8 million cases of TB were estimated in 2017 in India that makes quarter of the world’s TB cases,[2] and TB is still a serious threat against children and it is estimated to be 10% of the global incidence and 250,000 death in 1.74 million in reported pediatric TB cases in 2016.[3]

The immune response against TB is a rather complex process and still emerging field of interest. The innate immune system exhibits initial immune defense against TB infection by pathogen recognition receptors[4,5] that activate expression of several immune genes, cytokines, and interleukins.[5] Macrophages are the primary host cell target for tubercle bacilli for its intracellular growth and survival. Immune-primed macrophages are responsible for the activation of both innate and acquired immune responses[6] to eliminate tubercle bacilli before establishment of persistent infection.[5]

Several lines of regulatory mechanisms are underlying to regulate gene expression in human genome with respect to environmental cues. Transcription factors (TFs) are responsible for the controlled activation of genes under any circumstances such as pathophysiology of disease in particular.[7,8] Transcriptional regulation of genes is crucial in cellular developmental process and it reshapes the transcription program of immune cells under infection particularly.[7]

Alu repeat elements are present up to 1.4 million copies in human genome that comprises 11% of the total size.[8,9] Alu repeats are 300-nucleotide long repeats which belong to SINE family and retrotranspositionally inactive.[9,12] Due to its ample presence in human genome, these are normally found in gene-enriched regions and 3'UTR regions.[12] Alu and AluS are the main subtypes in Alu family[11] that has several TF-binding sites, namely, MEF2 and ATF families and LXR and RAR nuclear receptors. These are the important receptor families which are consistent with macrophage response against stress and infection conditions.[9] Furthermore, Alu

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repeats contain certain common transcriptional motif that is recognized by various TFs such as SP1, p53, c-MYC, ANRIL, and NF-B.[8,11] Several reports suggest that DNA methylation in Alu repeats was associated with several diseases. The hypomethylation status of Alu repeats supports its retrotransposition from one place to another to disrupt active gene body and also lead to Alu-mediated recombination. It was reported that Alu retrotransposition contributes about 0.3% of all human diseases.[13]

In the present study, we used the methylation-specific polymerase chain reaction (MS-PCR) method to perceive the methylation status of Alu repeat elements in genomic DNA isolated from whole blood from TB patients compared to healthy controls.

**Methods**

**Ethics statement**
The study protocol was approved by the Scientific Advisory Committee and Institute Ethical Committees. Blood samples were collected from TB-infected children and healthy donors after obtaining written informed consent.

**Patients and specimen collection**
Children with TB disease (<14 years, both genders) were recruited after obtaining informed consent. The inclusion criteria of both pulmonary TB (PTB) and extra-PTB (EPTB) patients included cartridge-based nucleic acid amplification test positive, smear positive, culture positive, chest X-ray positive, skin test positive, and clinical findings of TB disease. The inclusion criteria for healthy donors (<14 years, both genders) included no signs of TB and other infections, HIV negative, no surgical procedures underwent recently, asthma, and no other lung problems.

**Genomic DNA isolation**
Totally, 36 TB patients and 32 healthy controls were included in the study. Blood was collected from both groups and whole genomic DNA was isolated by FavorPrep Genomic DNA Mini Kit (Favorgen Biotech Corp., Taiwan) according to manufacturer’s procedure. Briefly, 300 µl of whole blood was used to isolate genomic DNA according to the manufacturer’s instructions. About 50 µl of elution buffer was used to elute DNA and stored at −20°C. NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) was used to measure the concentration of genomic DNA.

**Bisulfite conversion**
Bisulfite conversion of genomic DNA was followed according to EZ DNA Methylation-Gold Kit (Zymo Research Inc., USA) manufacturer’s procedure. Briefly, 500 ng of genomic DNA from both cases and controls was used for bisulfite chemical conversion to differentiate unmethylated C to U and methylated C remains unchanged. Finally, 15 µl of elution buffer was used to elute bisulfite converted DNA and stored at −20°C.

**Methylation standard DNA preparation**
Fully methylated (M) and fully unmethylated (U) control DNA was purchased commercially (QIAGEN, Germany). M and U bisulfite-converted DNAs were mixed to obtain a panel of DNA standards, i.e. 100%, 75%, 50%, 25%, 10%, and 0% by combining the proportions of M and U human control DNA [Supplementary Table 1].

**CpG Island location and primer design**
Supplementary Figure 1 shows the arrangement of consensus DNA sequence of Alu family and the location of CpG sites and CpG island (CGI) present in the given sequence. CGI in Alu repeats was identified by DBCAT tool (http://dbcat.cgm.ntu.edu.tw/), and MS-PCR primers were designed by MethPrimer tool with default settings.[14]

**Methylation-specific polymerase chain reaction**
Separate PCR reaction was performed specific for M and U MS-PCR analysis. A 20 µl of PCR mixture was prepared with 1 µl of bisulfite-converted DNA, 1 µl of each forward and reverse primers (10 pmol) from both methylated and unmethylated primers (separate reaction), 8 µl of ZymoTaq master mix, and 9 µl of nuclelease-free water. PCR cycle reaction was performed with initial denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing [Supplementary Table 2] for 35 s, and extension at 72°C for 30 s and a final extension at 72°C for 10 min.

**Agarose gel electrophoresis**
PCR product was separated in 1.5% agarose gel electrophoresis, and the intensity of the products and presence/absence of both M and U MS-PCR products were captured in ImageQuant LAS 500 (GE Healthcare, UK) and compared with control standards.

**Statistical analysis**
Categorical data were expressed as numbers and percentages. Percentage of Alu DNA methylation level was expressed in median with interquartile range (IQR) and Mann–Whitney t-test was performed to evaluate the difference of Alu DNA methylation level between cases and controls. Receiver operating characteristic (ROC) curve analysis was performed to evaluate the cutoff value of % Alu DNA methylation in the study groups. All statistical analyzes were carried out in SPSS v19 and MS Excel at 95% confidence interval with \( P < 0.05 \) was considered as statistically significant.

**Results**
Among the 36 cases, 18 (50%) were PTB and 18 (50%) were EPTB cases, and frequency of breakdown of EPTB cases enrolled in this study is represented in Figure 1. Baseline characteristics of the TB cases and healthy children enrolled in this study is represented in Table 1.

**Methylation of Alu elements in whole blood from tuberculosis patients and controls**
Methylation-specific primers were successfully designed for Alu repeat elements, and methylation level of Alu repeats
in whole-blood-derived DNA from TB-infected and healthy children was analyzed by MS-PCR. Methylation standard was prepared with mixture of both M and U DNA and subjected to MS-PCR procedure, and gel image was captured [Figure 2A line a]. This image was used to analyze and calculate the % Alu DNA methylation level in cases and controls.

Significant difference in Alu DNA methylation level was observed between cases and healthy controls. The median of Alu methylation observed in cases was 30% (IQR, 25–30%) and in controls was 75% (IQR, 50–75%) (P < 0.0001) [Figure 2B]. This shows that control samples were hypermethylated Alu repeats than TB cases [Figure 2A line b and B]. Occurrence of DNA methylation in Alu repeat sequences was observed both in cases and controls [Figure 2A lines b and c]. Hypermethylation was observed in healthy controls [Figure 2A line b] and hypomethylation was observed in TB cases [Figure 2A line c].

Correspondingly, MS-PCR products for unmethylated PCR reaction were run paralleled and documented. Percentage of methylation and unmethylation was calculated by comparing the PCR products with methylation standards that run paralleled. Some of the cases and controls showed difference in Alu methylation level by MS-PCR. Some of the cases were observed only methylated (hypomethylated) bands in gel with complete absence of unmethylated bands [Figure 2A lines d and e]. No significant difference was seen in Alu DNA methylation level compared among cases with different diagnostic procedures [Table 2], whereas significant difference in Alu DNA methylation level was observed in cases with different diagnostic criteria when compared with healthy controls [Table 3].

**Methylation of Alu elements in whole-blood pulmonary and extrapulmonary tuberculosis patients**

To further determine the level of Alu DNA methylation difference between PTB and EPTB infection in children, MS-PCR analysis was done in all PTB and EPTB cases. PTB cases showed a median of Alu methylation level as 30% (IQR, 25–30%) and EPTB cases showed a median of Alu methylation level as 27.5% (IQR, 21.25%–30%). Significant difference in Alu methylation was observed in PTB cases when compared to controls [Table 2], whereas no significant difference in Alu methylation was observed between PTB and EPTB cases [Table 3]. Relatively, PTB cases were methylated than EPTB cases, but no significant difference was observed (P = 0.5) [Table 3]. Among EPTB cases, median of Alu methylation in TB meningitis cases (n = 10) was 27.5% (IQR, 10%–35%) versus others were 27.5% (IQR, 25–30%) (P = 0.9) [Table 3].

**Diagnostic performance of DNA methylation level for tuberculosis disease**

ROC curve was constructed to assess the feasibility of Alu DNA methylation for the diagnosis of TB patients [Figure 3]. We analyzed the methylation level of Alu in whole-blood-derived DNA between TB patients and healthy controls. The area under the curve (AUC) was 0.969 (95% confidence interval, 0.936–1) (P < 0.0001).

### Table 1: Baseline characteristics of the enrolled children

| Characteristics      | Cases n=36 | Controls n=32 |
|----------------------|------------|---------------|
| Age (Years)          | 11 (7 Mon-14) | 9 (3-14)      |
| Sex (male and female)| 20+16      | 22+10         |
| Height (cm)          | 145 (63-150) | 126.8 (74-159)|
| Weight (kg)          | 16 (5.4-33)  | 26.3 (12-40)  |
| BCG scar (%)         | 36 (100)    | 32 (100)      |
| GeneXpert positive (%)| 8 (22.2) | Nil           |
| TST positive (%)     | 8 (22.2)    | Nil           |
| Culture positive (%) | 11 (30.5)   | Nil           |
| Type of TB (%)       |            |               |
| Pulmonary            | 18 (50)     | Nil           |
| Extrapulmonary       | 18 (50)     | Nil           |
| Fever (days)         | 7 (2-30)    | Nil           |
| Cough (days)         | 14 (6-30)   | Nil           |
| Chest pain (days)    | 6 (0-15)    | Nil           |
| Loss of appetite (days) | 10 (2-20) | Nil           |

TB: Tuberculosis, BCG: Bacillus Calmette–Guérin, TST: Tuberculin skin test

Figure 1: Distribution of tuberculosis disease in study children (n = 36)

Figure 2: Methylation-specific polymerase chain reaction results. (A) Comparison of whole-blood Alu DNA methylation levels between tuberculosis patients and controls. (B) Representative image methylation-specific polymerase chain reaction products. M indicates the fully methylated DNA used to prepare percentage proportion in methylation-specific polymerase chain reaction procedure. (a) Gel image of percentage of standard mDNA. (b) Methylation-specific polymerase chain reaction result of healthy controls. (c) Methylation-specific polymerase chain reaction result of tuberculosis cases. (d) Methylation-specific polymerase chain reaction result of tuberculosis cases – hypomethylation. (e) Unmethylation-specific methylation-specific polymerase chain reaction results of tuberculosis cases.
Percentage in Alu DNA methylation (median with IQR)

| Diagnostic test         | Percentage in Alu DNA methylation (median with IQR) | P     |
|-------------------------|------------------------------------------------------|-------|
| Cases (n=36)            | 30 (10-50)                                           | <0.0001|
| Controls (n=32)         | 75 (50-75)                                           |       |
| Pulmonary (n=18)        | 10 (25-30)                                           |       |
| Controls (n=32)         | 75 (50-75)                                           |       |
| Extrapulmonary (n=18)   | 10 (21.25-30)                                        |       |
| Controls (n=32)         | 75 (50-75)                                           |       |
| AFB positive (n=11)     | 10 (25-30)                                           |       |
| Controls (n=32)         | 75 (50-75)                                           |       |
| GeneXpert positive (n=8)| 5 (13.75-50)                                         |       |
| Controls (n=32)         | 75 (50-75)                                           |       |
| Meningitis (n=10)       | 27.5 (10-35)                                         | 0.5   |
| Controls (n=32)         | 75 (50-75)                                           |       |
| TST positive (n=8)      | 30 (30-45)                                           | 0.9   |
| Controls (n=32)         | 75 (50-75)                                           |       |

AFB: Acid-fast bacilli, TST: Tuberculin skin test, IQR: Interquartile range

ROC results indicate that the methylation level of Alu in whole blood may have good diagnostic value with 100% sensitivity and 84% specificity at the optimal cutoff value of 40% in Alu DNA methylation.

**DISCUSSION**

Human genome exhibits hypomethylation in most CGIs in common to maintain its open chromatin state to influence nearby gene expression. On the contrary, hypermethylation was observed in repetitive elements (REs), such as LINEs, SINEs, and LTRs to avert its own transcription and transposition to maintain genome integrity.\(^{[15,16]}\) Human REs make up more than half of the human genome that covers approximately 50% of all CpG dinucleotides in the human genome.\(^{[16,17]}\) Thus, it is being a widely used method of averaging the methylation level of RE for global DNA methylation analysis.\(^{[16]}\) Transcriptional regulation in eukaryotes maintains genome integrity and timely expression of genes required for developmental process. In mammals, the occurrence of methylation at the 5-C position of CpG dinucleotide in genomic DNA is an important epigenetic modification.\(^{[15]}\) Impact of DNA methylation seen in gene expression, embryonic development, differentiation, genome imprinting, transposon silencing/activation, aging, and carcinogenesis is well documented.\(^{[17,18]}\)

The manifestation of DNA Methylation in Alu repeats from whole blood was analyzed both in cases and controls. No difference was observed between the disease condition and gender category. No significant difference in Alu methylation level was seen with clinical parameters included in this study [Table 3]. Both PTB and EPTB cases were hypomethylated than control samples [Table 2]. Four PTB and EPTB cases (hypomethylated) showed complete absence of unmethylation products, and three PTB and EPTB showed 10% Alu methylation products by MS-PCR.

Variation in the level of Alu DNA methylation was observed among cases (IQR, 25–30%); on the other hand, controls showed hypermethylation in Alu repeat elements (IQR, 50–75%). The variation among cases might be due to the disease condition, level of disease progression, genetic backup, type of disease, and organism specific. Hypermethylation in healthy controls indicates that the appearance of more methylation of cytosine residues in Alu DNA elements than TB cases. The occurrence of hypomethylation of Alu repeats in TB cases may play a substantial role in disease progression/containment in the host. The hypomethylation of Alu repeats in TB-infected patients widen the possibilities of appropriate expression of immune genes to control TB infection. On the contrary, it may influence the expression of immunosuppressive or anti-inflammatory genes to aid tubercle bacilli to forfend host immune response.

*Others represents that the particular test either negative or not performed with its representative TB diagnostic test. AFB: Acid-fast bacilli, TST: Tuberculin skin test, IQR: Interquartile range, TB: Tuberculosis
by unbalancing the immune response at post-TB infection. Hypomethylation of Alu elements was implicated in a variety of diseases, but its role in TB infection is not yet understood completely.

Recently, H3K4 monomethylation (H3K4 me1) of active enhancer regions were studied in THP-1 cells by ChIP assay and revealed that around 40% of the de novo regions analyzed which contained the presence of Alu repeat elements, especially enriched in AluJ and AluS subunits of Alu family. Interestingly, these de novo H3K4 me1 peaks were associated with genes mainly involving in host defense and apoptosis of infected cells primarily macrophages. This indicates the importance of Alu repeat elements in reshaping the transcriptional program of infected cells and early immune response of macrophages against TB infection in the host.[8] This result indicates that hypomethylation of Alu/Alu-associated elements is linked to TB disease prevention in infected cells, and our results also support the notion that hypomethylation of Alu elements may influence the expression of immune genes responsible for TB containment in host.

ROC was generated to scrutinize the diagnostic accuracy of Alu DNA methylation in whole blood between cases and healthy controls. A cutoff value of 40% of Alu DNA methylation was observed to differentiate TB cases from healthy controls with 100% sensitivity and 84% specificity. ROC curve confirmed that detection of Alu methylation by MS-PCR assay is a good diagnostic tool (AUC = 0.969) in TB infection in children and hence proved that Alu methylation quantification performed in this study has the ability to differentiate children with TB infection and healthy controls effectively.

**Conclusion**

In the present study, hypomethylation of Alu repeat elements was observed in whole-blood genomic DNA derived from TB cases than healthy controls and demonstrated that detection of Alu methylation level may serve as a potential diagnostic and prognostic biomarker for TB infection in children. Although other gold standard TB diagnostic methods are still available with precise diagnostic potential, this Alu methylation detection also may have a good diagnostic value in future, and Alu hypomethylation might reflect the severity of TB disease in children. Further research should focus to identify and target specific CpG sites or CGI methylation by advanced technologies to improve the efficiency of the diagnostic method.

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**Conflicts of interest**

There was no conflict of interest.

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### Supplementary Table 1: Preparation of methylation standards of known methylation percentage

| Percentage of methylation standard | Positive control DNA (100 ng/µl) | Negative control DNA (100 ng/µl) |
|-----------------------------------|----------------------------------|----------------------------------|
| 0                                 | 0 µl                             | 10 µl                            |
| 10                                | 1.0 µl                           | 9.0 µl                           |
| 25                                | 2.5 µl                           | 7.5 µl                           |
| 50                                | 5.0 µl                           | 5.0 µl                           |
| 75                                | 7.5 µl                           | 2.5 µl                           |
| 100                               | 10.0 µl                          | 0 µl                             |

M: Methylation-specific primer, U: Unmethylation-specific primer, Tm: Melting temperature, F: Forward primer, R: Reverse primer, bp: Base pair in length

### Supplementary Table 2: Methylation-specific polymerase chain reaction primer details

| Gene | Primer type | Tm (°C) | CG%  | “C”s | Sequence (5’-3’)               | bp  |
|------|-------------|---------|------|------|------------------------------|-----|
| Alu  | M           | F       | 53   | 58.33| CGGATTATTTGAGGTAGGAGGTTTC     | 203 |
|      | R           |         | 68.0 | 9    | CCAAACACTAAAAATACAATAACCCTAT |     |

R: Reverse primer

U: Forward primer

bp: Base pair in length

### Supplementary Figure 1: Alu - consensus sequence and methylation-specific polymerase chain reaction primer design. The Alu sequence contains 282 bases, and the CpG Island is marked by light blue shading. The original input Alu DNA sequence showed in thick red line at the bottom, the level of methylation in CpG Island showed in guanine-cytosine (CG) percentage in the left side. Position of methylation-specific polymerase chain reaction primers was marked on the box and primers set-1 was selected with default settings.