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Global DNA hypomethylation of Alu and LINE-1 transposable elements as an epigenetic biomarker of anti-tuberculosis drug-induced liver injury

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ABSTRACT

Despite being highly effective, anti-tuberculosis (TB) drugs often induce adverse liver injury, anti-TB drug-induced liver injury (ATDILI), leading to treatment failure given no sensitive and selective ATDILI markers. Herein, we conducted a case–control association study to determine whether global DNA methylation of Alu and LINE-1 transposable elements responsible for genomic stability and transcriptional regulation was correlated with clinical parameters indicating ATDILI in TB patients and might serve as an ATDILI biomarker. Alu and LINE-1 methylation levels in blood leukocyte of 130 TB patients (80 ATDILI cases and 50 non-ATDILI cases) and 100 healthy controls were quantified using quantitative combined bisulfite restriction analysis. Both TB patients with and without ATDILI had significantly lower methylation levels of Alu and LINE-1 elements than healthy controls. Compared with non-ATDILI patients, Alu methylation levels were significantly decreased in ATDILI patients, commensurate with LINE-1 methylation analysis. Hypomethylation of Alu and LINE-1 measured within 1–7 days of TB treatment was independently associated with raised levels of serum aminotransferases assessed within 8–60 days of TB treatment. Receiver operating characteristic curve analysis uncovered that Alu and LINE-1 methylation levels were both more sensitive and specific for differentiating ATDILI cases from non-ATDILI cases than serum aminotransferases after starting TB treatment within 1–7 days. Kaplan-Meier analysis displayed a significant association between hypomethylation of Alu and LINE-1 elements and an increased rate of ATDILI occurrence in TB patients. Collectively, global DNA hypomethylation of Alu and LINE-1 elements would reflect ATDILI progression and might serve as novel sensitive and specific ATDILI biomarkers.

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Introduction

Tuberculosis (TB) is the most common life-threatening opportunistic infection caused by the bacterium *Mycobacterium tuberculosis*, which is becoming a major global health problem [1]. Although the first-line TB treatment with rifampicin, isoniazid, pyrazinamide, and ethambutol has been shown to quell the increasing incidence of TB, the concurrent treatment commonly leads to the occurrence of severe adverse events [2]. One of the most frequent and serious adverse effects observed during TB treatment is hepatotoxicity (also known as anti-TB drug-induced liver injury, ATDILI), leading to poor medication adherence and eventually to treatment failure in TB patients [3,4]. In serious cases, ATDILI can ultimately result in acute liver failure, in which some patients need liver transplantation for long-term survival, thereby posing a significant challenge to the early control of TB progression. In that context, early and accurate detection of ATDILI is crucial to improving TB control. Current diagnosis of ATDILI is accompanied by liver function biomarkers, particularly alanine aminotransaminase (ALT) regarded as a gold standard for determining liver injury. However, it has been reported that elevated levels of ALT are not specific to DILI [5] and can produce false positive results owing to metabolic perturbations [6,7]. From the aforementioned challenges, it is important to identify specific and mechanistic biomarkers of ATDILI, which may pave the way for improving treatment outcomes of TB. Regarding this matter, a better understanding of causes

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associated with ATDILI would yield important insights into mechanistic biomarkers for ATDILI.

Of various risk factors known to be involved in ATDILI, genetic polymorphisms of pharmacogenes potentially influencing their expressions have been reported to affect drug response and toxicity [8–11]. Nonetheless, the occurrence of ATDLI cannot be completely identified by DNA variations. In the past decade, an increasing number of studies have attempted to link epigenetic marks, especially DNA methylation within promoter regions of metabolic enzymes-encoding genes to ATDILI development [12–15]. DNA methylation, one of epigenetic mechanisms by which DNA base cytosine (C) is converted to 5-methylcytosine (5-mC) by DNA methyltransferases, plays an important role in modulating gene expression without changing DNA sequence, resulting in decreased transcriptional expression or even gene repression [16]. Indeed, a large portion of differentially methylated CpG sites within the genome are normally found in transposable repetitive elements including Alu (also known as short-interspersed nuclear elements, SINE) and long-interspersed nuclear element (LINE-1), both of which are reportedly associated with total genomic methylation content [17], thus serving as a proxy for global DNA methylation. Hypomethylation of those transposable elements has been shown to stimulate their retrotransposition activity potentially contributing to genomic instability and deregulation of gene expression [18]. Clear evidence from clinical studies uncovered relationships between hypomethylation of Alu and LINE-1 elements and various pathological conditions – especially liver injuries [19–21]. On the basis of previous findings, it is reasonable to speculate that DNA methylation measured within Alu and LINE-1 elements may be associated with ATDILI development and could be used as an ATDILI biomarker.

To the extent of our knowledge, no attempt has been made to capture the breadth of relationship between global DNA methylation and ATDILI. Consequently, the purpose of this study was to determine whether global DNA methylation of Alu and LINE-1 elements was associated with clinical parameters indicating ATDILI and could be used as an early biomarker for predicting and monitoring ATDILI progression in TB patients.

Materials and methods

The study protocol was approved by the Institutional Review Board of the Faculty of Dentistry/Faculty of Pharmacy, Mahidol University (IRB number 2018/061.1110) and was conducted in congruence with the guidelines of the Declaration of Helsinki. All participants were fully informed regarding the study protocols and procedures. Written informed consent was acquired from all subjects prior to their enrollment in this study.

Study subjects

This multicenter case–control study included 130 TB patients diagnosed by clinical blood tests, a simple skin test, as well as histological findings and 100 healthy controls. The healthy controls who attended an annual health check-up at Chiang Rai Prachanukroh Hospital and had no clinical indicators and symptoms of TB or other medical conditions such as autoimmune or liver diseases based on medical records and consultations were enrolled through convenience sampling. All TB patients from the 10 designated hospitals (Bangplama Hospital, Suphan Buri; The Central Chest Disease Institute, Nonthaburi; Chiang Rai Prachanukroh Hospital, Chiang Rai; Hatyai Hospital, Songkla; Maesot Hospital, Tak; Nopparat Rajathanee Hospital, Bangkok; Buddhachinaraj Hospital, Phitsanulok; Ramathibodi Hospital, Bangkok; Rayong Hospital, Rayong; and Thai Mueang Chaipat Hospital, Phang-nga) received short-course anti-TB drugs consisting of rifampicin (8–12 mg/kg once-daily dosing), isoniazid (4–6 mg/kg once-daily dosing), pyrazinamide (20–30 mg/kg once-daily dosing), and ethambutol (15–25 mg/kg once-daily dosing) for the first 2 months followed by rifampicin (8–12 mg/kg once-daily dosing) and isoniazid (4–6 mg/kg once-daily dosing) for the next 4 months, according to World Health Organization guidelines [22,23]. In terms of hepatotoxicity, all recruited TB patients were categorized into those with ATDILI ($n = 80$) and non-ATDILI ($n = 50$) groups with regard to their blood levels of liver function tests. In compliance with clinical practice guidelines for TB treatment in Thailand [24], ATDILI cases met at least one of the following criteria: (i) aspartate aminotransferase (AST) and ALT elevations above 5-fold the upper limit of normal (ULN); (ii) AST and/or ALT elevations above 3-fold ULN along with one symptom of hepatitis including anorexia, fatigue, nausea, vomiting, jaundice, liver enlargement and/or dark urine; or (iii) AST and/or ALT elevations along with total bilirubin elevation above 3-fold ULN with or without symptoms of hepatitis. Patients with other hepatic diseases, such as viral hepatitis or chronic liver dysfunction, those who exhibited abnormal liver function tests at baseline, and those who received other known hepatotoxic drugs were excluded from the study.

Peripheral blood samples were drawn from healthy controls and TB patients who were treated with anti-TB drugs within 1–7 days. Liver function tests including AST, ALT, alkaline phosphatase (ALP), total

Table 1. Clinical characteristics of TB patients with and without ATDILI after commencement of TB treatment.

Variables	Within 1–7 days of treatment		*P*-value	Within 8–60 days of treatment		*P*-value
	ATDILI ($n = 80$)	Non-ATDILI ($n = 50$)		ATDILI ($n = 80$)	Non-ATDILI ($n = 50$)	
Age (years)	53.50 (36.50-66.00)	43.00 (33.00, 60.50)	0.089	53.50 (36.50, 66.00)	43.00 (33.00, 60.50)	0.089
Gender (F/M)	46.25% / 53.75%	28.00% / 72.00%	0.029	46.25% / 53.75%	28.00% / 72.00%	0.029
BMI (kg/m^2)	18.63 (16.34, 21.41)	20.06 (17.34, 21.17)	0.375	18.63 (16.34, 21.41)	20.06 (17.34, 21.17)	0.375
ALT (IU/L)	29.50 (18.50, 95.75)	24.00 (14.00, 33.00)	0.702	109.00 (54.00, 165.00)	20.00 (15.00, 34.00)	**<0.001**
AST (IU/L)	30.00 (13.00, 65.00)	31.00 (23.00, 44.00)	0.554	162.00 (97.00, 303.00)	24.00 (19.50, 34.00)	**<0.001**
ALP (IU/L)	112.00 (86.00, 168.00)	73.00 (62.00, 107.50)	**0.021**	137.00 (108.25, 177.00)	95.50 (74.25, 124.38)	**0.007**
Total bilirubin (mg/dL)	0.90 (0.60, 1.63)	0.36 (0.30, 0.59)	**<0.001**	1.50 (0.80, 3.16)	0.50 (0.38, 0.80)	**<0.001**
Direct bilirubin (mg/dL)	0.40 (0.10, 0.88)	0.13 (0.10, 0.26)	0.236	0.85 (0.40, 2.82)	0.22 (0.10, 0.38)	**0.004**

Note: Data are represented as either median with interquartile ranges (IQR) for continuous variables or percentages for categorical variables. *P*-values marked with bold indicate statistically significant differences between the groups.
Abbreviations: ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ATDILI: anti-tuberculosis drug-induced liver injury; BMI: body mass index; F: female; M: male; TB: tuberculosis.

bilirubin, and direct bilirubin are routinely performed by an automated machine.

Quantification of global DNA methylation

DNA methylation of Alu and LINE-1 elements was quantitated using quantitative combined bisulfite restriction analysis (qCOBRA), as previously detailed [20]. Briefly, genomic DNA was extracted from peripheral blood leukocyte of the study subjects using the QIAamp DNA Blood Mini Kit (Qiagen, CA, USA) following the manufacturer's protocol. Extracted DNA (50 ng) was treated with sodium bisulfite using EZ DNA Methylation Gold Kit (Zymo Research, Orange, CA, USA), according to the manufacturer's protocol. The bisulfite-converted DNA was subsequently amplified by PCR with primers specific to Alu and LINE-1 elements. After PCR amplification, Alu amplicons were treated with 2 U *TaqI* in *TaqI* buffer (MBI Fermentas, Burlington, Canada), whereas LINE-1 amplicons were digested with 2 U *TaqI* and 8 U *TasI* in NEBuffer 3 (New England Biolabs, Ontario, Canada). Afterwards, the digested fragments were incubated at 65 °C overnight and then separated by an 8% non-denaturing polyacrylamide gel stained with ethidium bromide. Finally, band intensities were analysed by gel documentation with GeneTools analysis software (SYNGENE, Cambridge, UK) to estimate the percent methylation of repetitive elements. For all experiments, DNA samples extracted from HeLa, Jurkat, and Daudi cell lines were used as positive controls to normalize inter-assay variations.

Statistical analysis

All statistical analyses were carried out by the statistical package for social sciences version 26.0 (SPSS, Inc., Chicago, IL, USA). Differences in demographic and clinical characteristics between groups were estimated using chi-square (χ^2) test for categorical variables represented as percentages and Mann Whitney *U* test for continuous variables represented as median with interquartile ranges (IQR, Q1-Q3). Comparing continuous variables described as median with IQR among groups were executed using Kruskal–Wallis *H* test. Association between 2 variables was analysed using Spearman's rho correlation. To control the effects of confounding factors including age, sex, and body mass index (BMI) on the outcome of interest, a multivariate regression analysis was undertaken. Diagnostic accuracy of biomarkers for ATDILI was evaluated using construction of a receiver operating characteristic (ROC) curve. To determine the feasible use of global DNA methylation as a prognostic marker for ATDILI progression, Kaplan-Meier curves were drawn for all patients grouped into low- and high methylation levels of Alu or LINE-1 elements based

on the optimal cut-off point derived from ROC curve analysis. A *P*-value of less than 0.05 was considered statistically significant for all analyses.

Results

Clinical characteristics of TB patients with and without ATDILI

Baseline demographic and clinical characteristics of TB patients with and without ATDILI are detailed in Table 1. After commencement of TB treatment within 1–7 days, there were no significant differences in age, BMI, and liver function tests including ALT, AST, and direct bilirubin between TB patients with and without ATDILI, whereas there was a significantly higher percentage of male patients in the non-ATDILI group (72.00%) than in the ATDILI group (53.75%) ($P < 0.029$). Besides this, ALP and total bilirubin were observed to be significantly higher in ATDILI cases than those in non-ATDILI cases ($P = 0.021$, $P < 0.001$, respectively). After starting treatment within 8–60 days, TB patients with ATDILI had significantly increased serum levels of ALT, AST, ALP, total bilirubin, and direct bilirubin when compared with those with non-ATDILI ($P < 0.001$, $P < 0.001$, $P = 0.007$, $P < 0.001$, $P = 0.004$, respectively). Considering baseline demographic characteristics of TB patients and healthy controls, median age, gender ratio, and median BMI in TB patients [48.00 (35.00, 58.50); 57 (43.85%) females and 73 (56.15%) males; 19.50 (16.65, 21.19) kg/m^2; respectively] and healthy controls [49.00 (34.50, 54.50); 31 (31.00%) females and 69 (69.00%) males; 19.07 (17.32, 20.31) kg/m^2; respectively] were not significantly different, as described in Supplementary table 1.

Global DNA hypomethylation of Alu and LINE-1 elements in TB patients with ATDILI

As depicted in Figure 1(A), median percentage of Alu methylation was significantly lower in TB patients with ATDILI than that in those without ATDILI and healthy controls ($P < 0.001$, $P < 0.001$, respectively). In TB patients without ATDILI, median percentage of Alu methylation was significantly decreased, compared to healthy controls ($P < 0.001$, $P < 0.001$, respectively) (Figure 1(A)). Consistent with Alu methylation analysis, LINE-1 methylation levels were found to be significantly reduced in both TB patients with and without ATDILI when compared to healthy controls ($P < 0.001$, $P < 0.001$, respectively) (Figure 1(B)). Compared with TB patients without ATDILI, the patients with ATDILI exhibited significantly decreased LINE-1 methylation levels ($P < 0.001$) (Figure 1(B)).

As Alu and LINE-1 are both transposable elements accounting for almost 50% of the human genome, we further examined a possible correlation between Alu and LINE-1 methylation levels. We also found a close link between Alu and LINE-1 methylation levels (Spearman's Rho correlation coefficient = 0.61, $P < 0.001$) (Figure 1(C)), thereby attesting the potential utility of DNA methylation measured within both elements as a surrogate marker for global DNA methylation.

Global DNA hypomethylation of Alu and LINE-1 elements as an independent determinant of ATDILI

Given that there was a significant difference in gender ratio between TB patients with and without ATDILI, we performed multivariate logistic regression analysis to examine whether global DNA hypomethylation of Alu and LINE-1 elements was an independent risk factor of ATDILI. Association between global DNA methylation of Alu and LINE-1 elements and ATDILI is shown in Table 2. After adjusting for age, gender, and BMI, Alu methylation levels in TB patients with ATDILI remained significantly lower than in non-ATDILI patients (odd ratios, OR = 0.81; 95% CI: 0.747, 0.889; $P < 0.001$), consistent with LINE-1 methylation analysis (OR = 0.87; 95% CI: 0.817, 0.923; $P < 0.001$). Using the optimal cut-off value derived from ROC curve analysis, Alu and LINE-1 methylation levels were both categorized into hypomethylation (Alu <52.8%, $n = 65$; LINE-1 < 73.7%, $n = 65$) and hypermethylation (Alu $\geq$ 52.8%, $n = 65$; LINE-1 $\geq$ 73.7%, $n = 65$). Multivariate logistic regression analysis with adjustment for the above confounders revealed that TB patients with Alu hypomethylation exhibited a significantly elevated risk of ATDILI, compared with those with Alu hypomethylation (OR = 4.89; 95% CI: 1.981, 12.090; $P = 0.001$). In parallel with Alu methylation analysis, subsequent analysis unveiled that LINE-1 hypomethylation was significantly associated with a 9.72-fold increased risk of ATDILI in TB patients, compared with LINE-1 hypermethylation (OR = 9.72; 95% CI: 2.587, 36.489; $P = 0.001$).

Negative associations between Alu and LINE-1 methylation levels and clinicopathological parameters in TB patients

Due to global DNA hypomethylation in TB patients with ATDILI, we subsequently determined whether Alu and LINE-1 methylation levels were related to clinicopathological parameters indicating ATDILI progression in TB patients. Relationships between Alu and LINE-1 methylation levels measured within 1–7 days of treatment initiation and clinical

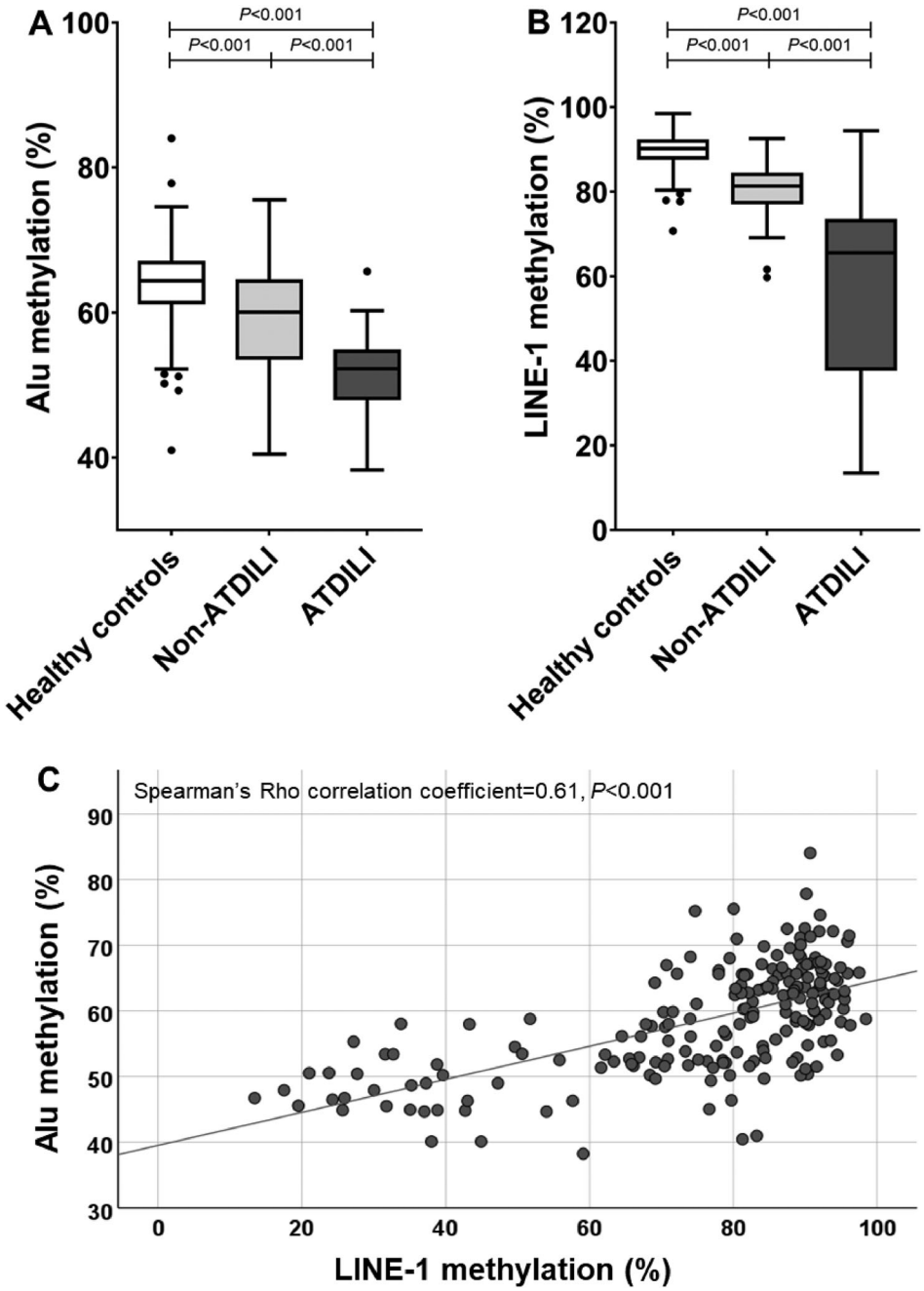


Figure 1. Global DNA methylation of Alu and LINE-1 elements in healthy controls and TB patients. (A) Alu methylation in blood leukocyte of heathy controls and TB patients with and without ATDILI. (B) LINE-1 methylation in blood leukocyte of heathy controls and TB patients with and without ATDILI. (C) Close link between Alu and LINE-1 methylation levels in all recruited participants.

parameters assessed within either 1–7 days or 8–60 days of starting treatment in TB patients are demonstrated in Figure 2. Spearman's Rho correlation analysis unveiled negative associations of Alu methylation levels with serum AST and total bilirubin in TB patients who were treated with a TB regimen within 1–7 days of initiation (Spearman's Rho correlation coefficient = −0.26, $P = 0.038$; Spearman's Rho correlation coefficient = −0.28, $P = 0.046$, respectively). Furthermore, Alu methylation levels quantified within 1–7 days after treatment were found to be inversely associated with serum aminotransferases detected after treatment within 8–60 days including ALT (Spearman's Rho correlation coefficient = −0.31, $P = 0.001$) and AST (Spearman's Rho correlation coefficient = −0.33, $P = 0.001$). Apart from this, there were significantly inverse correlations between LINE-1 methylation levels and liver function tests including ALT (Spearman's Rho correlation coefficient = −0.31, $P = 0.012$), AST (Spearman's Rho correlation coefficient = −0.29, $P = 0.023$), ALP (Spearman's Rho correlation coefficient = −0.36, $P = 0.008$), total bilirubin (Spearman's Rho correlation coefficient = −0.46, $P = 0.001$), and direct bilirubin (Spearman's Rho correlation coefficient = −0.35, $P = 0.047$) in TB patients after treatment initiation within 1–7 days. LINE-1

Table 2. Multivariate logistic regression analysis of associations between Alu as well as LINE-1 methylation levels and ATDILI.

Variables	TB patients with and without ATDILI	
	OR (95%CI)	*P*-value[a]
Alu elements		
Methylation levels	0.81 (0.747, 0.889)	<0.001
Methylation status		
• Hypomethylation	4.89 (1.981, 12.090)	0.001
• Hypermethylation	Reference	
LINE-1 elements		
Methylation levels	0.87 (0.817, 0.923)	<0.001
Methylation status		
• Hypomethylation	9.72 (2.587, 36.489)	0.001
• Hypermethylation	Reference	

Note: *P*-values marked with bold indicate statistically significant differences between the groups. Abbreviations: ATDILI: anti-tuberculosis drug-induced liver injury; Alu: short-interspersed nuclear elements; LINE-1: long-interspersed nuclear element; TB: tuberculosis.
[a]Adjusted for age, gender, and BMI.

methylation levels measured within 1–7 days of treatment were also observed to be negatively correlated with liver function tests measured after starting treatment within 8–60 days including ALT (Spearman's Rho correlation coefficient = −0.49, $P < 0.001$) and AST (Spearman's Rho correlation coefficient = −0.47, $P < 0.001$).

We further evaluated independent associations of clinical parameters with decreases in Alu and LINE-1 methylation levels using multivariate linear regression analysis with adjusting for age, gender, BMI, and unrelated clinical parameters consisting of ALP, total bilirubin, and direct bilirubin. As displayed in Table 3, a reduction in Alu methylation levels measured within 1–7 days of treatment was independently associated with increased serum levels of AST and ALT assessed after initiating treatment within 8–60 days in TB patients (β-coefficient = −0.018; 95% CI: −0.034, −0.002; $P = 0.032$; β-coefficient = −0.030; 95% CI: −0.055, −0.004; $P = 0.025$, respectively). In addition to this, further analysis showed that decreased LINE-1 methylation levels quantified within 1–7 days of treatment initiation were independently correlated with increased serum levels of ALT estimated after starting treatment within 8–60 days in TB patients (β-coefficient = −0.028; 95% CI: −0.056, −0.001; $P = 0.044$) (Table 4).

Alu and LINE-1 methylation levels as early biomarkers for AIDILI

The area under the ROC curve (AUC) was calculated to identify the potential usefulness of global DNA methylation as a biomarker for early progression of AIDILI in TB patients. As revealed in Figure 3(A),

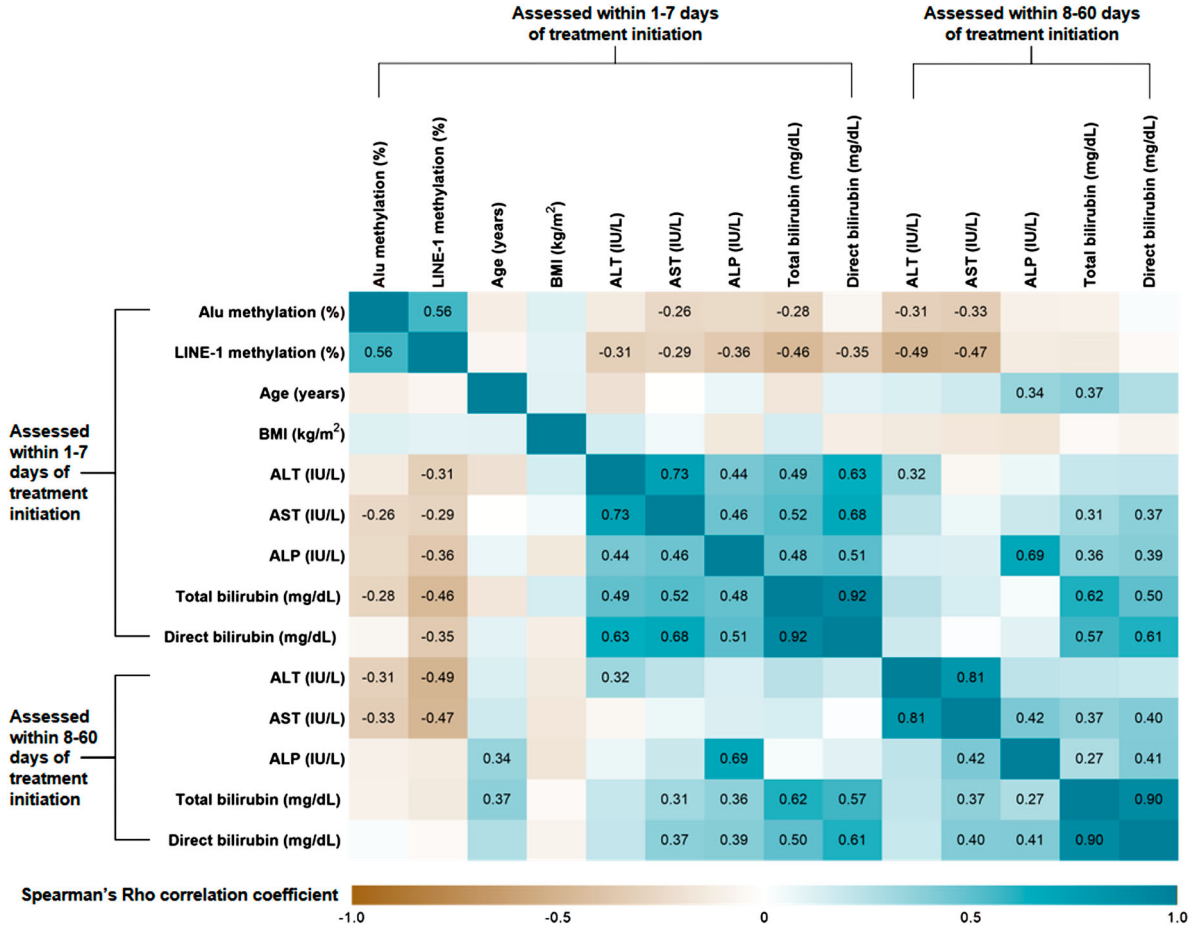


Figure 2. Heatmap of Spearman's Rho correlations between Alu and LINE-1 methylation levels and clinical parameters indicating ATDILI progression in TB patients. Significant correlation coefficients were shown in matrix correlation heatmap.

Table 3. Multivariate linear regression analysis of associations between Alu methylation levels measured within 1–7 days of treatment and clinical parameters assessed within either 1–7 days or 8–60 days of treatment initiation in TB patients.

Variables	Alu methylation levels (%)	
	β-coefficient (95% CI)	*P*-value[a]
Within 1–7 days of treatment		
Age (years)	−0.091 (−0.322, 0.141)	0.234
Gender (F/M)	−1.453 (−15.615, 12.709)	0.702
BMI (kg/m^2)	−0.204 (−2.012, 1.603)	0.675
ALT (IU/L)	−0.008 (−0.026, 0.010)	0.369
AST (IU/L)	−0.005 (−0.044, 0.033)	0.783
ALP (IU/L)	−0.003 (−0.026, 0.019)	0.753
Total bilirubin (mg/dL)	0.421 (−2.001, 2.842)	0.723
Direct bilirubin (mg/dL)	1.860 (−0.029, 3.750)	0.053
Within 8–60 days of treatment		
Age (years)	−0.039 (−0.242, 0.163)	0.678
Gender (F/M)	−4.135 (−11.020, 2.750)	0.213
BMI (kg/m^2)	−0.413 (−1.155, 0.329)	0.246
ALT (IU/L)	−0.030 (−0.055, −0.004)	**0.025**
AST (IU/L)	−0.018 (−0.034, −0.002)	**0.032**
ALP (IU/L)	−0.006 (−0.019, 0.007)	0.373
Total bilirubin (mg/dL)	−0.391 (−1.080, 0.297)	0.256
Direct bilirubin (mg/dL)	−0.537 (−1.449, 0.376)	0.237

Note: *P*-values marked with bold indicate statistically significant associations. Abbreviations: ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ATDILI: anti-tuberculosis drug-induced liver injury; BMI: body mass index; F: female; M: male.
[a]Adjusted for age, gender, BMI, ALP, total bilirubin, and direct bilirubin.

ROC curve analysis uncovered that after starting TB treatment within 1–7 days, Alu methylation levels were significantly more sensitive and specific for differentiating ATDILI cases from non-ATIDLI cases than a combination of serum ALT and AST in TB patients. Regarding this, Alu methylation values of 52.8% provided a sensitivity of 89.7%, a specificity of 73.5%, and an AUC of 0.94 (95% CI: 0.879, 0.992; $P < 0.001$), while the AUC of ALT combined with AST was not statistically significant.

Table 4. Multivariate linear regression analysis of associations between LINE-1 methylation levels measured within 1–7 days of treatment and clinical parameters assessed within either 1–7 days or 8–60 days of treatment initiation in TB patients.

Variables	LINE-1 methylation levels (%)	
	β coefficient (95% CI)	*P*-value[a]
Within 1–7 days of treatment		
Age (years)	−0.025 (−0.077, 0.027)	0.105
Gender (F/M)	−10.995 (−9.873, −1.118)	0.107
BMI (kg/m^2)	0.680 (−0.691, 2.051)	0.100
ALT (IU/L)	0.004 (−0.032, 0.040)	0.805
AST (IU/L)	0.021 (−0.034, 0.075)	0.436
ALP (IU/L)	−0.014 (−0.108, 0.080)	0.756
Total bilirubin (mg/dL)	1.734 (−1.979, 5.446)	0.335
Direct bilirubin (mg/dL)	4.719 (−2.079, 11.517)	0.134
Within 8–60 days of treatment		
Age (years)	0.114 (−0.241, 0.470)	0.485
Gender (F/M)	−8.234 (−29.329, 12.861)	0.400
BMI (kg/m^2)	0.954 (−0.297, 2.205)	0.119
ALT (IU/L)	−0.028 (−0.056, −0.001)	**0.044**
AST (IU/L)	−0.012 (−0.031, 0.006)	**0.183**
ALP (IU/L)	−0.011 (−0.033, 0.012)	0.344
Total bilirubin (mg/dL)	−0.377 (−1.382, 0.628)	0.451
Direct bilirubin (mg/dL)	−0.328 (−1.934, 1.279)	0.677

Note: *P*-values marked with bold indicate statistically significant associations. Abbreviations: ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ATDILI: anti-tuberculosis drug-induced liver injury; BMI: body mass index; F: female; M: male.
[a]Adjusted for age, gender, BMI, ALP, total bilirubin, and direct bilirubin.

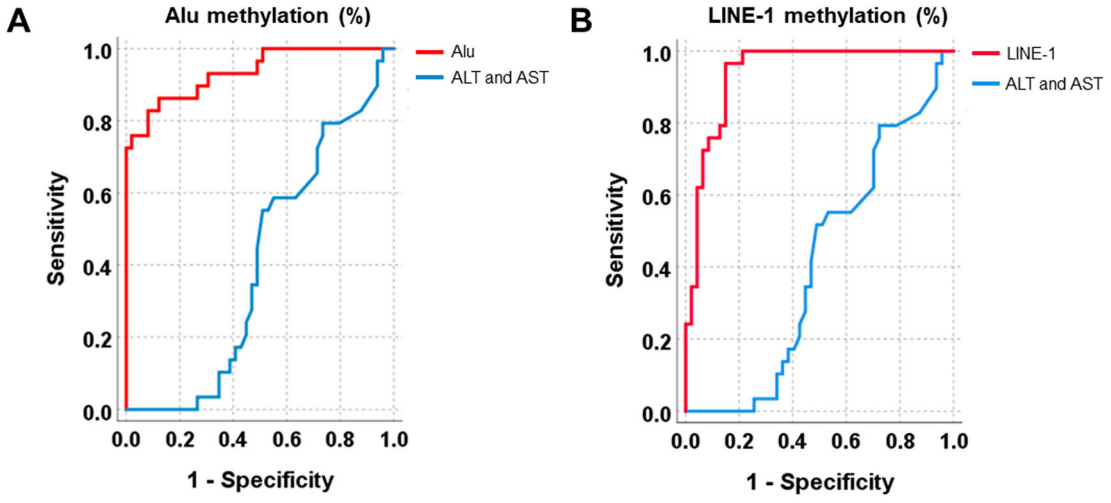
Based on LINE-1 methylation assessed within 1–7 days of treatment, the optimal cut-off value for discriminating TB patients with ATDILI from those with non-ATDILI was defined at 73.7%, which yielded a sensitivity of 96.6%, a specificity of 85.1%, and an AUC of 0.94 (95% CI: 0.888, 0.991; $P < 0.001$) (Figure 3(B)). In contrast to this, after treatment initiation within 1–7 days, the AUC of serum ALT combined with AST for distinguishing ATDILI patients with from non-ATIDLI patients was not statistically significant (Figure 3(B)). The above findings indicate that LINE-1 methylation levels quantified within 1–7 days of treatment were more sensitive and selective for identifying early progression of ATDILI than a combination of serum aminotransferases measured within 1–7 days of treatment.

Association between decreased methylation levels of Alu and LINE-1 and an increased rate of ATDILI occurrence

To evaluate the effect of hypomethylation of Alu and LINE-1 elements on cumulative rates of ATDILI occurrence in TB patients, Kaplan-Meier analysis was additionally undertaken. For Alu methylation levels, TB patients with Alu hypomethylation (75.4%) had a significantly greater cumulative rate of ATDILI occurrence than those with Alu hypermethylation (47.7%) (log-rank: $\chi^2 = 15.02$, $P < 0.001$) (Figure 4(A)). Comparably, a significant increase in cumulative rate of ATDILI occurrence was detected in TB patients with LINE-1 hypomethylation (92.4%), compared with those with LINE-1 hypermethylation (30.8%) (log-rank: $\chi^2 = 49.87$, $P < 0.001$) (Figure 4(B)).

Discussion

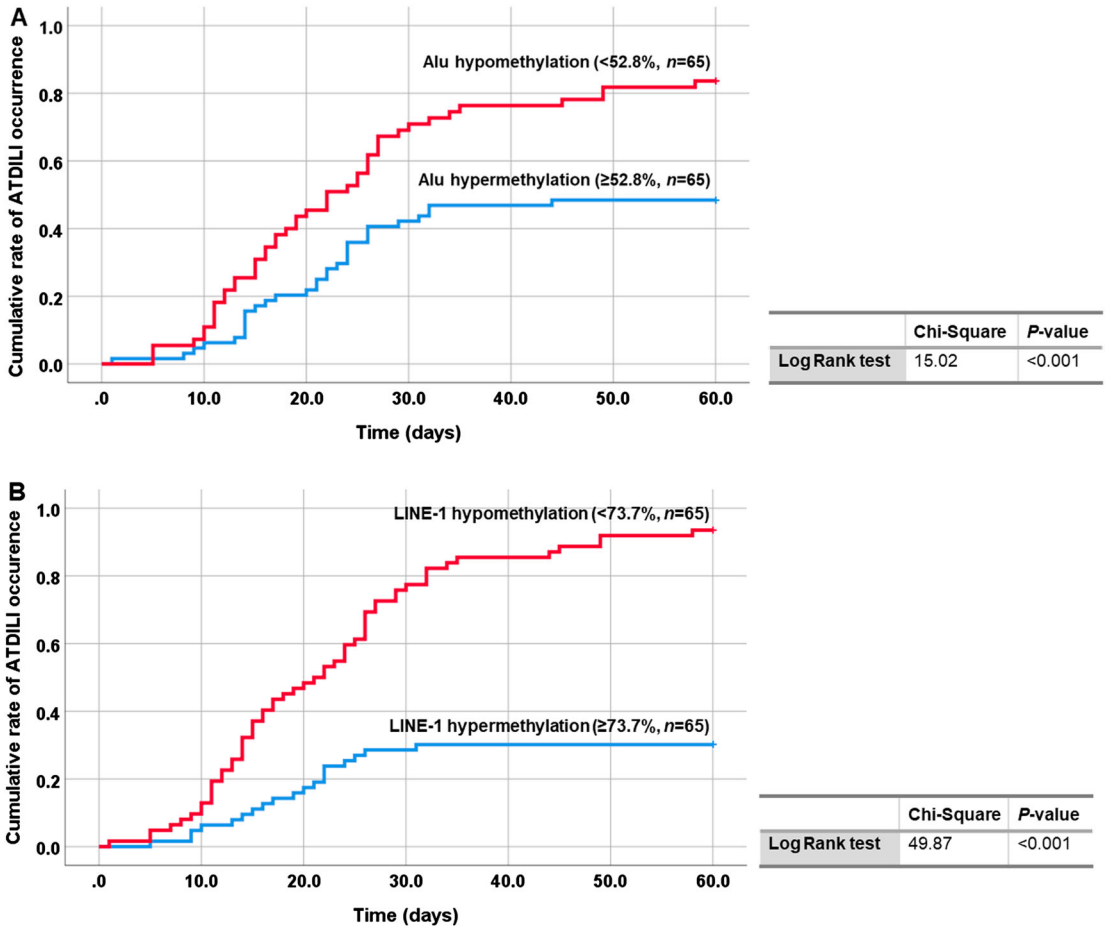
As alterations in the expression and activity of drug metabolic enzymes are well-recognized as pathological features driving the developmental and progressive ATDILI in TB patients, it is noteworthy that better understanding of factors influencing aberrant expression of molecules relevant to ATDILI may hold great promise for identifying mechanistic biomarkers for ATDILI. Although genetic variants have been shown to affect transcriptional regulation of gene products related to hepatotoxic mechanisms [25], these risk factors are unable to fully explain a high absolute risk of ATDILI in individual patients due to the pathogenic complexity of ATDILI. It seems likely that non-specific genetic factors may help explain the remaining variation of ATDILI occurrence in individual patients and refine our knowledge of ATDILI. DNA methylation, an epigenetic mechanism allowing integration of genetic and environmental factors to regulate gene expression, is suspected to be an alternative factor associated with ATDILI. In



Variables detected within 1-7 days	AUC	95% CI	*P*-value
Alu	0.935	0.879, 0.992	<0.001
ALT and AST	0.596	0.279, 0.529	0.160

Variables detected within 1-7 days	AUC	95% CI	*P*-value
LINE-1	0.939	0.888, 0.991	<0.001
ALT and AST	0.589	0.284, 0.538	0.171

Figure 3. ROC curve showing the potential utility of Alu and LINE-1 methylation levels as diagnostic biomarkers for ATDILI in TB patients. (A) Alu methylation as an early biomarker for distinguishing ATDILI cases from non-ATDILI cases. (B) LINE-1 methylation as an early biomarker for distinguishing ATDILI cases from non-ATDILI cases.



	Chi-Square	*P*-value
Log Rank test	15.02	<0.001

	Chi-Square	*P*-value
Log Rank test	49.87	<0.001

Figure 4. Kaplan-Meier curve for the occurrence of ATDILI in TB patients. (A) Significant association between Alu hypomethylation and an increased rate of ATDILI occurrence. (B) Significant association between LINE-1 hypomethylation and an increased rate of ATDILI occurrence.

support of this hypothesis, a number of clinical studies demonstrated that altered DNA methylation levels within promoter regions of several metabolic enzymes-encoding genes including *CYP2E1*, *CYP2D6*, *GSTP1*, and *NAT2* were associated with ATDILI development in TB patients [14,15,26]. In addition to this, a genome-wide association study of DNA methylation provided a supporting finding of a correlation between altered DNA methylation levels within various genes and ATDILI progression [12]. On the basis of the previous findings, we aimed to measure Alu and LINE-1 methylation levels considered a proxy for global DNA methylation in blood leukocyte of TB patients with and without ATDILI within 1–7 days of treatment initiation compared to healthy controls and also found global DNA hypomethylation in TB patients with ATDILI. Furthermore, global DNA hypomethylation of Alu and LINE-1 elements was found to be independently associated with an increased risk of ATDILI in TB patients. More specifically, decreased Alu and LINE-1 methylation levels were detected to be associated with increased levels of serum aminotransferases assessed after starting treatment within 8–60 days in TB patients. From our findings, it has been postulated that global DNA hypomethylation of Alu and LINE-1 elements may be implicated in the developmental and progressive ATDILI. Despite no previous studies revealing a direct relationship between global DNA hypomethylation and ATDILI, there are considerable published data on Alu and LINE-1 hypomethylation in a wide range of chronic liver injuries [19–21], which attests to our findings. Altogether, the aforementioned findings led us to consider the hypothesis that global DNA methylation may have potential as an epigenetic biomarker for early detection of ATDILI. To address the postulation, our further results derived from ROC curve analysis demonstrated that Alu and LINE-1 methylation levels measured within 1–7 days after starting TB treatment were both more sensitive and specific for discriminating TB patients with ATDILI from those with non-ATDILI than serum aminotransferases detected after initiating treatment within 1–7 days. Alongside this, our additional analysis unveiled a positive correlation between global DNA hypomethylation and an increased rate of ATDILI occurrence in TB patients. Taken together, all foregoing findings shed light on the utility of epigenetic biomarkers, especially global DNA methylation for predicting and monitoring ATDILI progression in TB patients.

In the view of our findings above, even though the exact mechanism underlying global DNA hypomethylation in ATDILI has not yet been fully elucidated, the possible reason for hypomethylation of Alu and LINE-1 elements may be a result of excessive reactive oxygen species (ROS) accumulation-induced high oxidative stress that can alter DNA methylation levels and eventually lead to cellular damage. This hypothesis is supported by an experimental study demonstrating that anti-TB drugs can cause increased ROS production-induced hepatocellular damage in mice [27]. Correspondingly, high oxidative stress has been reported to provoke global DNA hypomethylation [28], resulting in activation of transposable elements including Alu and LINE-1 to change their position within the human genome and ultimately altering genome size and gene expression [29,30]. These phenomena may help explain why Alu and LINE-1 elements were significantly hypomethylated in TB patients – especially in those with ATDILI.

In spite of significant findings presented herein, some inherent limitations should be taken into consideration. The first drawback is the fact that investigation on global DNA methylation in tissue-specific liver cells of TB patients with ATDILI was unachievable. However, it has been documented that to some extent, methylation levels of a certain tissue can be determined from peripheral blood [31]. Based on this premise, it is conceivable that Alu and LINE-1 methylation levels in blood leukocyte may reflect alterations in their methylation levels in the liver. Another caveat is lack of data on oxidative stress that makes it difficult to determine whether global DNA hypomethylation is associated with high oxidative stress in TB patients with ATDILI. Likewise, primarily given lack of data on biochemical parameters of healthy controls recruited in this study, it is difficult to ensure whether global DNA hypomethylation is associated with increased levels of serum aminotransferases in non-pathological conditions. In a similar manner, due to unavailability of data on comorbidities associated with ATDILI, it is challenging to interpret our finding that global DNA hypomethylation of Alu and LINE-1 elements was independently associated with an elevated rate of AIDILI occurrence in TB patients. Additionally, since this study is cross-sectional in its design with a relatively small number of participants, unequivocal conclusions on the causal relationships between global DNA methylation and ATDILI development in TB patients cannot be drawn.

To sum up, this study is the first to provide novel evidence revealing hypomethylation of Alu and LINE-1 elements in blood leukocyte of TB patients – especially those with ATDILI. More precisely, decreases in Alu and LINE-1 methylation levels measured within 1–7 days of starting TB treatment were independently associated with increases in serum aminotransferases detected after commencement of treatment within 8–60 days in TB patients. Particularly, ROC curve analysis showed that both Alu and LINE-1 methylation levels were more sensitive and selective for differentiating ATDILI cases from non-ATDILI cases than serum

aminotransferases in TB patients after starting treatment within 1–7 days. Supporting those findings, Kaplan-Meier analysis depicted that TB patients with global DNA hypomethylation had a significantly increased rate of ATDILI occurrence compared with those with global DNA hypermethylation, thus highlighting the potential usefulness of global DNA methylation as a prognostic biomarker for ATDILI in TB patients. Collectively, DNA methylation of Alu and LINE-1 elements, considered a surrogate marker for global DNA methylation, appears to have potential as a sensitive and specific biomarker for early ATDILI progression in TB patients after initiating treatment within 1–7 days. To verify the feasible use of global DNA methylation as an epigenetic biomarker for ATDILI, future validation with a prospective cohort study is needed.

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Disclosure statement

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References

[1] Doherty AM, Kelly J, McDonald C, et al. A review of the interplay between tuberculosis and mental health. Gen Hosp Psychiat. 2013;35:398–406.
[2] McIlleron H, Meintjes G, Burman WJ, et al. Complications of antiretroviral therapy in patients with tuberculosis: drug interactions, toxicity, and immune reconstitution inflammatory syndrome. J Infect Dis. 2007;196:S63–S75.
[3] Yee D, Valiquette C, Pelletier M, et al. Incidence of serious side effects from first-line antituberculosis drugs among patients treated for active tuberculosis. Am J Respir Crit Care Med. 2003;167:1472–1477.
[4] Gulbay BE, Gurkan OU, Yildiz OA, et al. Side effects due to primary antituberculosis drugs during the initial phase of therapy in 1149 hospitalized patients for tuberculosis. Respir Med. 2006;100:1834–1842.
[5] Senior JR. Alanine aminotransferase: a clinical and regulatory tool for detecting liver injury–past, present, and future. Clin Pharmacol Ther. 2012;92:332–339.
[6] Hanley AJ, Williams K, Festa A, et al. Elevations in markers of liver injury and risk of type 2 diabetes: the insulin resistance atherosclerosis study. Diabetes. 2004;53:2623–2632.
[7] Sattar N, Scherbakova O, Ford I, et al. Elevated alanine aminotransferase predicts new-onset type 2 diabetes independently of classical risk factors, metabolic syndrome, and C-reactive protein in the west of Scotland coronary prevention study. Diabetes. 2004;53:2855–2860.
[8] Wang L, McLeod HL, Weinshilboum RM. Genomics and drug response. N Engl J Med. 2011;364:1144–1153.
[9] Evans WE, McLeod HL. Pharmacogenomics–drug disposition, drug targets, and side effects. N Engl J Med. 2003;348:538–549.
[10] Daly AK. Drug-induced liver injury: past, present and future. Pharmacogenomics. 2010;11:607–611.
[11] Nanashima K, Mawatari T, Tahara N, et al. Genetic variants in antioxidant pathway: risk factors for hepatotoxicity in tuberculosis patients. Tuberculosis. 2012;92:253–259.
[12] Huai C, Wei Y, Li M, et al. Genome-wide analysis of DNA methylation and antituberculosis drug-induced liver injury in the Han Chinese population. Clin Pharmacol Ther. 2019;106:1389–1397.
[13] Li Y, Li Y, Zheng G, et al. Cytochrome p450 1A1 and 1B1 promoter CpG island methylation regulates rat liver injury induced by isoniazid. Mol Med Rep. 2018;17:753–762.
[14] Zhang J, Zhu X, Li Y, et al. Correlation of CpG island methylation of the Cytochrome P450 2E1/2D6 genes with liver injury induced by anti-tuberculosis drugs: a nested case-control study. Int J Environ Res Public Health. 2016;13:776.
[15] He L, Gao L, Shi Z, et al. Involvement of cytochrome P450 1A1 and glutathione S-transferase P1 polymorphisms and promoter hypermethylation in the progression of anti-tuberculosis drug-induced liver injury: a case-control study. PLoS One. 2015;10:e0119481.
[16] Portela A, Esteller M. Epigenetic modifications and human disease. Nat Biotechnol. 2010;28:1057–1068.
[17] Weisenberger DJ, Campan M, Long TI, et al. Analysis of repetitive element DNA methylation by MethyLight. Nucleic Acids Res. 2005;33:6823–6836.
[18] Gaudet F, Hodgson JG, Eden A, et al. Induction of tumors in mice by genomic hypomethylation. Science. 2003;300:489–492.
[19] Zheng Y, Hlady RA, Joyce BT, et al. DNA methylation of individual repetitive elements in hepatitis C virus infection-induced hepatocellular carcinoma. Clin Epigenetics. 2019;11:145.
[20] Udomsinprasert W, Kitkumthorn N, Mutirangura A, et al. Global methylation, oxidative stress, and relative telomere length in biliary atresia patients. Sci Rep. 2016;6:26969.

[21] Anwar SL, Hasemeier B, Schipper E, et al. LINE-1 hypomethylation in human hepatocellular carcinomas correlates with shorter overall survival and CIMP phenotype. PLoS One. 2019;14:e0216374.
[22] World Health Organization (WHO). Guidelines for treatment of drugsusceptible tuberculosis and patient care. Geneva: WHO Press; 2017. https://doi.org/10.1586/17476348.1.1.85.
[23] World Health Organization (WHO). Latent tuberculosis infection: updated and consolidated guidelines for programmatic management. Geneva: WHO Press; 2018. https://doi.org/10.1056/NEJMcp021045.
[24] Services, Do.M. Clinical practice guideline of tuberculosis treatment in Thailand 104, (2018).
[25] Pachkoria K, Lucena MI, Crespo E, et al. Analysis of IL-10, IL-4 and TNF-alpha polymorphisms in drug-induced liver injury (DILI) and its outcome. J Hepatol. 2008;49:107–114.
[26] Zhang D, Hao J, Hou R, et al. The role of NAT2 polymorphism and methylation in anti-tuberculosis drug-induced liver injury in Mongolian tuberculosis patients. J Clin Pharm Ther. 2020;45:561–569.
[27] Sharma V, Kaur R, Sharma VL. Ameliorative potential of Adhatoda vasica against anti-tubercular drugs induced hepatic impairments in female Wistar rats in relation to oxidative stress and xeno-metabolism. J Ethnopharmacol. 2021;270:113771.
[28] Furlan D, Trapani D, Berrino E, et al. Oxidative DNA damage induces hypomethylation in a compromised base excision repair colorectal tumourigenesis. Br J Cancer. 2017;116:793–801.
[29] Zhang Z, Saier MH Jr. A novel mechanism of transposon-mediated gene activation. PLoS Genet. 2009;5:e1000689.
[30] Bourque G, Burns KH, Gehring M, et al. Ten things you should know about transposable elements. Genome Biol. 2018;19:199.
[31] Lokk K, Modhukur V, Rajashekar B, et al. DNA methylome profiling of human tissues identifies global and tissue-specific methylation patterns. Genome Biol. 2014;15:r54.