Alu Hypomethylation As A Diagnostic Biomarker for Anti-Tuberculosis Drug-Induced Liver Injury

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Abstract

The first-line antituberculosis drug can cause hepatic injuries leading to acute liver failure and treatment discontinuation due to a lack of selective biomarker. Thus, the present study was designed to investigate whether Alu methylation was associated with anti-tuberculosis drug-induced liver injury (ATDILI) and could be an early ATDILI marker. Alu methylation levels in blood leucocyte of 47 tuberculosis patients with ATDILI, 48 tuberculosis patients with non-ATDILI, and 100 healthy controls were measured using quantitative combined bisulfite restriction analysis (qCOBRA). Methylation levels within Alu elements in the ATDILI cases were considerably reduced, compared with tuberculosis patients with non-ATDILI and healthy controls. In addition, hypomethylation of Alu was independently correlated with heightened susceptibility to ATDILI. Besides, multivariate linear regression analysis uncovered that Alu hypomethylation was independently associated with liver function indicators including aspartate transaminase and alanine transaminase evaluated within 60 days after initiation of the anti-tuberculosis drugs. Receiver-operating characteristic curve analysis revealed that Alu hypomethylation (AUC = 0.82) in peripheral blood leukocytes provided more specific and sensitive values than the original biomarkers such as aspartate transaminase and alanine transaminase, which cloud serve as a novel marker for differentiating tuberculosis patients with ATDILI from non-ATDILI within 7 days after treatment with the anti-tuberculosis drugs. Accordingly, Alu hypomethylation in blood leucocytes could be used as a novel biomarker for early ATDILI development.

Keywords**:** *Alu, Anti-tuberculosis drug-induced liver injury, Liver function indicators, Biomarker*

1. Introduction

Mycobacterium tuberculosis (MTB) is an infectious human pathogen that infects the respiratory tract. M. tuberculosis has usually an effect on the lungs causing a chronic respiratory condition or medically called pulmonary tuberculosis (TB) (Salvatore & Zhang, 2017). Concerning the proclamation of World Health Organization (WHO), pulmonary TB is the main cause of public health problems leading to mortality worldwide of global communities (Organization, 2020). Most patients with pulmonary TB exhibited clinical symptoms including sputum production, weight loss, chronic cough, night sweats, appetite loss, hemoptysis, and fever (Zumla, Raviglione, Hafner, & von Reyn, 2013). Currently, the intensive regimen for TB treatment includes rifampicin (RIF), isoniazid (INH), pyrazinamide (PZA), and ethambutol (EMB) (Nahid et al., 2016). Nevertheless, previous studies have reported that first-line antituberculosis drugs including RIF, INH, and PZA are frequently causative of idiosyncratic liver injury (Saukkonen et al., 2006; Huang, 2014; Chowdhury et al., 2006). Antituberculosis drug-induced liver injury (ATDILI) contributes to substantial liver failure and fatality and reduces treatment success (Tostmann et al., 2008). Therefore, in the period of disease treatment, the monitoring and appraisal of liver injury are imperative to prevent liver injury from the standard antituberculosis drugs (Saukkonen et al., 2006). Traditional diagnosis of drug-induced liver injury (DILI) is the monitoring and evaluation of increased serum in enzyme activities of aminotransferases and bilirubin levels (Björnsson, 2006), but these traditional biomarkers are not specific for detecting the early DILI development and progression. According to Moggs, Goodman, Trosko, and Roberts (2004) and Watson et al. (2004), the results derived from their previous studies have demonstrated that the capability of toxicants such as pharmaceutical agents can induce alterations in epigenetic levels. Remarkably, it has been suggested that modifying global DNA methylation levels and methylation levels within particular genes influenced by drug-induced epigenetic changes may be more selective and sensitive than traditional indicators for toxicity evaluation. Besides, they may have the potential as a clinical biomarker for ATDILI.

DNA methylation is considered one of three main epigenetic modifications, which regulates the expression of human genes by the addition of methyl-group at 5'- cytosine base within cytosine-phosphate-guanine (CpG) dinucleotide islands (Pogribny & Beland, 2009). Generally, the pattern of DNA methylation in the human genome is heritable; nonetheless, the DNA methylation pattern is susceptible to changes by several factors including age, gender, environment, and lifestyle (Gravina & Vijg, 2010). It has been suggested that the alterations in DNA methylation levels were associated with the pathogenesis in several pathological conditions and may be clinically employed as possible biomarkers for several diseases such as atherosclerosis, cancer, Alzheimer's disease, and psychiatric (Pogribny & Beland, 2009). Furthermore, aberrant DNA methylation levels have been reported to influence the expressions of over 60 genes associated with the absorption, distribution, metabolism, and excretion (ADME) of drugs in drug-induced toxicities (Fisel, Schaeffeler, & Schwab, 2016). For instance, a whole-genome DNA methylation study by Huai et al. (2019) has reported that patients with ATDILI showed abnormal *CYP* promoter methylation levels, and aberrant methylation levels within different genes, such as *AK2*, *SLC8A2*, and *PSTPIP2*, influence the cellular response in those patients receiving rifampicin treatment. Based on these previous findings, epigenetic changes, particularly DNA methylation may be associated with ATDILI etiology.

Given that transposon elements comprise at least 40% of methylation in the human genome, they were employed as a useful proxy for global DNA methylation measurement (Deininger, Moran, Batzer, & Kazazian, 2003). Generally, transposon elements consist of Alu elements or short interspersed nuclear elements (SINEs) and long interspersed nuclear elements (LINEs) accounting for 11% and 17% of the human genome, respectively (Deininger et al., 2003). In normal cells, transposon elements including Alu elements were methylated, which prevent transcriptional activation and retrotransposition. On the other hand, the hypomethylation of transposon elements reportedly induces retrotransposition of transposable elements, dysregulation of DNA repair genes (Bae et al., 2012), and altered expressions of important genes, leading to genomic instability (Gravina et al., 2010). Alu element is one of the transposon elements implicated in the etiology of several diseases--especially liver diseases (Kim, Cho, Han, & Lee, 2016). Furthermore, Alu hypomethylation has been reported to be associated with the progression of cholestatic liver disease (Udomsinprasert et al., 2016). Thus, the alterations in methylation status within Alu elements may be associated with ATDILI and could be used as a useful biomarker for an early ATDILI progression in tuberculosis patients.

2. Objectives

1) To evaluate Alu methylation in peripheral blood leukocytes from tuberculosis patients and healthy controls.

2) To compare Alu methylation in peripheral blood leukocytes between tuberculosis patients with and without ATDILI.

3) To investigate whether Alu methylation was related to clinical indicators indicating ATDILI in tuberculosis patients and may serve as biomarkers for ATDILI in tuberculosis patients.

3. Materials and Methods

3.1 Study participants

Following the guidelines of the proclamation of Helsinki, this study protocol was authorized by the Institutional Review Board of the Faculty of Dentistry/Faculty of Pharmacy, Mahidol University Participants provided written informed consent before their participation.

The study population was composed of 95 tuberculosis patients diagnosed by tuberculin skin test, tuberculosis blood test, as well as medical history and 100 age- and gender-matched healthy controls without a history of tuberculosis, hepatic diseases, or autoimmune were recruited in the current work. All tuberculosis patients received first-line antituberculosis drugs consisting of isoniazid, rifampicin, pyrazinamide, plus ethambutol. Tuberculosis patients with ATDILI were diagnosed with liver function indicators such as serum AST, ALT, and TB levels. Following clinical practice guideline for tuberculosis treatment in Thailand (Services, 2018), tuberculosis patients with ATDILI who 1) had serum ALT and/or AST levels higher than

three times the upper limit of normal (ULN) plus one symptom of hepatitis, including dark urine, anorexia, liver enlargement, fatigue, jaundice or nausea vomiting or 2) had serum ALT and AST levels higher than five times ULN or total bilirubin level higher than three times ULN with or without hepatitis conditions were considered. Participants who were diagnosed with liver cirrhosis, viral hepatitis, and metabolic syndromes as well as having a previous history of liver disease were excluded from this study. The patients in a program of medical treatment with some drug-induced liver injury including halothane, atenolol, fluconazole, methotrexate, labetalol, verapamil, phenytoin, quinine, phenobarbitone, quinidine, probenecid, carbamazepine, allopurinol, ethionamide, salicylates, valproate, cimetidine, and fluconazole were excluded.

The standard ethylenediaminetetraacetic acid (EDTA) tube was used to isolate leukocytes from peripheral blood samples obtained from tuberculosis patients after receiving standard regimens within 7 days and age-matched controls. Then, these samples were maintained at -20°C. Besides, Liver function indicators consisting of AST, ALT, ALP, TB, and DB were commonly evaluated by employing an automated machine.

3.2 Quantification of Alu methylation levels

DNA was isolated from blood leukocyte using a commercial DNA isolation kit (GE Healthcare, UK), and extracted DNA was then treated by EZ DNA Methylation Gold Kit (Zymo Research, Orange, CA, USA), based on the manufacturer's protocol. Alu methylation levels were assessed by quantitative combined bisulfite restriction analysis (qCOBRA). Alu methylation pattern was examined by 8% non-denaturing polyacrylamide gel, and the gel was stained with ethidium bromide, as previously described by Udomsinprasert et al., (2016). Band intensities were measured by GeneSys software.

3.3 Statistical analysis

Statistical analysis was carried out with the SPSS statistical package, version 18.0 (SPSS Inc., Chicago, IL). Comparison in mean was determined by Student's unpaired t-test, whereas comparison in abnormal distribution of Alu meth level was carried out by either Mann-Whitney U test (2 groups) or Kruskal-Wallis H test (more than 2 groups). Spearman's rho correlation was utilized to estimate correlations, and multivariate linear regression models were carried out for independent association between Alu methylation levels and clinical variables. The receiver operating characteristic (ROC) curve was constructed to appraise the utilization of Alu methylation levels as a potent biomarker for ATDILI progression. The data were presented as mean ± standard deviation (SD). All statistical tests were considered statistically significant with P-values of less than 0.05, based on the two-tailed probability.

4. Results and Discussion

4.1 Results

The clinicopathological variables of the study subjects were presented in Tables 1 and 2. No significant difference in mean age, gender ratio, and body mass index (BMI) between the healthy controls and tuberculosis patients were observed. Within 7 days after treatment with the standard regiments, the findings were not significantly different in characteristics and clinical indicators, including total bilirubin (TB), direct bilirubin (DB), alanine transaminase (ALT), alkaline phosphatase (ALP), and aspartate transaminase (AST) between the ATDILI cases and non-ATDILI cases. However, liver function indicators including TB, DB, ALT, and AST values detected within 60 days in the period of the treatment were significantly higher in the ATDILI cases than that in the non-ATDILI cases ($p = 0.02$, $p = 0.02$, $p < 0.001$, $p < 0.001$, respectively).

Table 1 Clinicopathological variables of tuberculosis patients with and without ATDILI estimated within 7 days after the initiation with first-line anti-tuberculosis drug regiment

Variables	Within 7 days after treatment ATDILI	Non-ATDILI	P value
Number	47	48	
Age (years)	51.11 ± 16.40	45.38 ± 15.51	0.83
Gender (F/M)	20 (42.6%)/27 (57.4%)	13 (27.1%)/35 (72.9%)	0.11
BMI (kg/m2)	19.20 ± 4.46	19.64 ± 2.96	0.64
TB (mg/dL)	1.10 ± 1.19	0.46 ± 0.30	0.08
DB (mg/dL)	0.48 ± 0.80	0.15 ± 0.04	0.51
ALT (IU/L)	55.19 ± 130.02	31.53 ± 22.31	0.49
ALP (IU/L)	131.90 ± 113.74	96.77 ± 58.04	0.31
AST (IU/L)	47.08 ± 88.71	34.80 ± 16.03	0.60

Note: Abbreviations: ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; ATDILI, anti-tuberculosis drug-induced liver injury; BMI, body mass index; DB, direct bilirubin; F, female; M, male; TB, total bilirubin

Table 2 Clinicopathological variables of tuberculosis patients with and without ATDILI estimated within 60 days after the initiation with first-line anti-tuberculosis drug regiment

Variables	Within 60 days after treatment ATDILI	Non-ATDILI	P value
Number	47	48	
Age (years)	51.11 ± 16.40	45.45 ± 15.51	0.83
Gender (F/M)	20 (42.6%)/27 (57.4%)	13 (27.1)/35 (72.9%)	0.11
BMI (kg/m2)	19.20 ± 4.46	19.64 ± 2.96	0.64
TB (mg/dL)	2.44 ± 3.50	0.64 ± 0.34	0.02
DB (mg/dL)	1.57 ± 2.33	0.30 ± 0.26	0.02
ALT (IU/L)	134.78 ± 99.54	26.53 ± 16.67	< 0.001
ALP (IU/L)	176.39 ±139.97	116.56 ± 83.68	0.12
AST (IU/L)	232.79 ± 209.13	27.74 ± 12.55	< 0.001

Note: Abbreviations: ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; ATDILI, anti-tuberculosis drug-induced liver injury; BMI, body mass index; DB, direct bilirubin; F, female; M, male; TB, total bilirubin

Subsequently, as presented in Figure 1, Alu methylation levels in TB patients with ATDILI was significantly reduced when compared with non-ATDILI tuberculosis patients (54.79 ± 2.95 vs 60.31 ± 5.68 %, $p < 0.001$) and the healthy control (54.79 ± 2.95 vs 63.98 ± 4.92 %, $p < 0.001$).

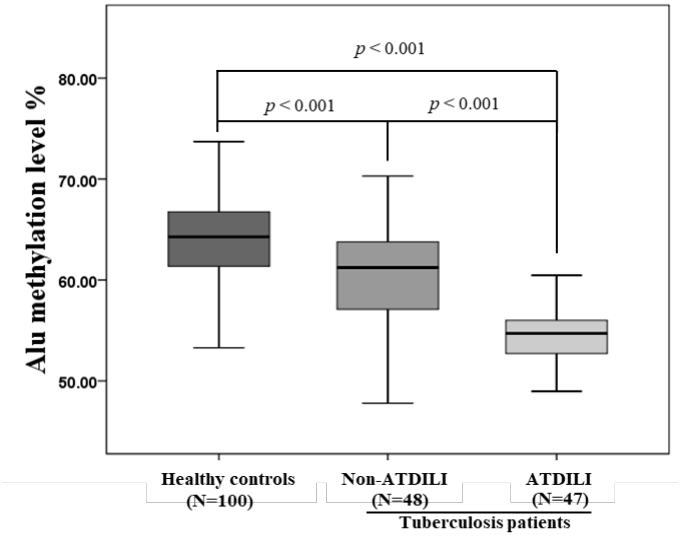


Figure 1 Blood leukocytes Alu methylation levels in both healthy controls and tuberculosis patients with and without ATDILI. Abbreviations: ATDILI, anti-tuberculosis drug-induced liver injury

The authors further examined the association between Alu methylation levels and clinical indicators in tuberculosis patients with and without ATDILI during the treatment of anti-tuberculosis drugs within 7 and 60 days. As presented in Table 3, Spearman's rho correlation analysis revealed that Alu methylation levels were negatively related to liver function indicators such as ALT (r = -0.45, $p < 0.001$) and AST (r = -0.52, $p < 0.001$) evaluated within 60 days in the period of the treatment in both TB patients with and without ATDILI. Furthermore, there was a significant inverse correlation between Alu methylation and gender (r = -0.29, $p = 0.005$).

Table 3 Spearman's rho correlation analyzes of Alu methylation level in tuberculosis patients with and without ATDILI associated with clinicopathological variables

Variables	Alu methylation % Spearman's rho correlation			
	Within 7 days after treatment		Within 60 days after treatment	
	Coefficient (r)	P value	Coefficient (r)	P value
Age (years)	-0.83	0.43	-0.83	0.43
Gender (F/M)	-0.29	0.005	-0.29	0.005
BMI (kg/m2)	-0.03	0.81	-0.03	0.81
TB (mg/dL)	-0.17	0.38	-0.12	0.47
DB (mg/dL)	0.10	0.71	-0.14	0.46
ALT (IU/L)	0.17	0.30	-0.45	< 0.001
ALP (IU/L)	-0.06	0.75	-0.14	0.37
AST (IU/L)	0.30	0.06	-0.52	< 0.001

Note: Abbreviations: ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; ATDILI, anti-tuberculosis drug-induced liver injury; BMI, body mass index; DB, direct bilirubin; F, female; M, male; TB, total bilirubin

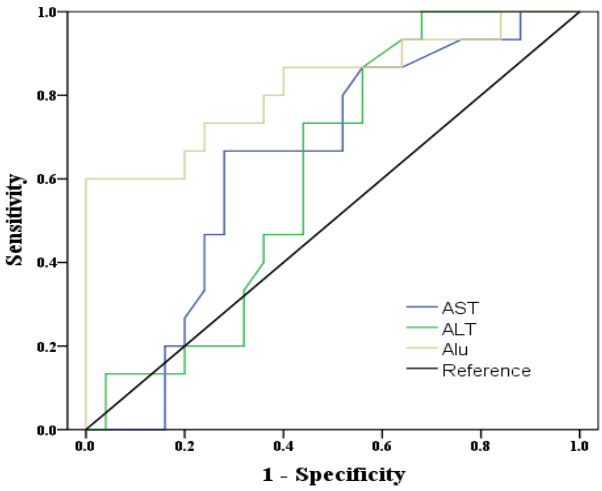
Additionally, the multivariate linear regression analysis showed that Alu methylation levels in blood leucocyte of both ATDILI and non-ATDILI cases were independently correlated with ALT (β-coefficient = -0.29; 95% CI: -0.030 to -0.006; $p = 0.004$) and AST (β-coefficient = -0.28; 95% CI: -0.015 to -0.002; $p = 0.006$), after adjustments with confounding factors including age and gender, as revealed in Table 4.

Table 4 Multivariate linear regression analyses of Alu methylation level in tuberculosis patients with ATDILI and without ATDILI

Variables	Alu methylation levels value	β coefficient (95% CI) P
Age (years)	-	-
Gender (F/M)	-0.30 (-5.779 to -1.234)	0.003
BMI (kg/m2)	-	-
DB (mg/dL)	-	-
TB (mg/dL)	-	-
ALT (IU/L)	-0.29 (-0.030 to -0.006)	0.004
ALP (IU/L)	-	-
AST (IU/L)	-0.28 (-0.015 to -0.002)	0.006

Note: Abbreviations: ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; BMI, body mass index; DB, direct bilirubin; F, female; M, male; TB, total bilirubin

The receiver operating characteristic (ROC curve) was created to reveal whether Alu hypomethylation in blood leucocyte cloud possibly is an indicator for ATDILI in the tuberculosis patients during treatment with the first-line antituberculosis drugs. The result demonstrated the area under curve (AUC) of Alu hypomethylation at 0.82 (95% CI: 0.68 to 0.97; p = 0.001), as presented in Figure 2. Besides, the optimal cutoff value of Alu hypomethylation was at 55.7%, which provided a sensitivity of 73% and a specificity of 72%. Inversely, there was no significant statistical difference in the AUC of ALT and AST values investigated within 7 days during treatment with the first-line antituberculosis drugs, suggesting methylation levels within Alu element showing more specific and selective than the conventional indicators, and serving as a potential indicator for distinguishing the tuberculosis patients with early ATDILI from the non-ATDILI.



	AUC	95% CI	*P-value*
Alu	0.82	0.676 to 0.967	0.001
ALT	0.62	0.441 to 0.788	0.230
AST	0.64	0.466 to 0.816	0.139

Figure 2 ROC curve analysis of Alu methylation level in the peripheral blood leukocyte as the biomarker for ATDILI

4.2 Discussion

Alu hypomethylation was found to be associated with poor prognosis in various human diseases (Ye, Jiang, Zhang, & Mao, 2020; Thongsroy, Patchsung, & Mutirangura, 2017; Jintaridth, Tungtrongchitr, Preutthipan, & Mutirangura, 2013; Chen et al., 2013). Even though the biological function of Alu remains unclear, several studies uncovered that Alu hypomethylation has an important role in genomic deletion and rearrangement (Kim et al., 2016). Based on its role, Alu hypomethylation has been proposed to be implicated in ATDILI, given that genetic defects (Yew, Chang, & Chan, 2018), and epigenetic changes (Huai et al., 2019, Zhang et al., 2016, He et al., 2015) reportedly contributed to ATDILI in some tuberculosis patients by overproduction of reactive oxygen species (ROS), leading to damage in hepatic cells in response to high oxidative stress (Yew et al., 2018, Russmann, Jetter, & Kullak-Ublick, 2010). High oxidative stress can cause cellular apoptosis and necrosis mediated through an immune system (Russmann et al., 2010). In addition, ROS overproduction promoted aberrant DNA methylation and induced a variety of DNA lesions contributing to several diseases (Birben, Sahiner, Sackesen, Erzurum, & Kalayci, 2012). For example, hypomethylation of retrotransposable DNA elements in peripheral blood leukocytes was associated with elevated oxidative DNA damage of biliary atresia (BA) patients (Udomsinprasert et al., 2016). In an animal study, anti-tuberculosis drugs consisting of rifampicin and isoniazid cloud generate ROS that promoted hepatocellular damage in mice (Chowdhury et al., 2006). From the above-mentioned findings, it had been supported that alterations in methylation status in transposon elements could be used as a clinical biomarker for the early progression of ATDILI in TB patients. To address these hypotheses, this was the first study to assess methylation levels of Alu in TB patients with ATDILI compared to those without ATDILI and healthy controls. In our study, a significant reduction in Alu methylation levels was found in TB patients with ATDILI, compared with TB patients without ATDILI (54.79 ± 2.95 vs 60.31 ± 5.68 %) and the healthy controls (54.79 ± 2.95 vs 63.98 ± 4.92 %). Consistent with previous studies, Alu hypomethylation was found to be associated with severity of type 2 diabetes mellitus patients (Thongsroy et al., 2017), post-menopausal women with osteoporosis (Jintaridth et al., 2013), and glioma patients (Chen et al., 2013). More precisely, our further findings showed that Alu methylation status in the TB patients with ATDILI and non-ATDILI cases was correlated with liver function indicators, including AST and ALT evaluated within 60 days after commencement of anti-TB treatment. In the ROC curve analysis, the result revealed that Alu hypomethylation had higher values of specificity, sensitivity, and AUC than those of classical indicators such as AST and ALT evaluated within 7 days after initiation of anti-TB treatment. It had been suggested that Alu hypomethylation was able to be a clinical biomarker for detecting the early development and progression of ATDILI.

Firstly, the limitation of this study was the fact that measurement of methylation level in tissue-specific liver cells of tuberculosis patients with ATDILI was limited, because of ethical consideration. Secondly, peripheral blood leukocytes contained a heterogeneous mixture of cell types, which could interfere with methylation levels. Thirdly, the modifications of DNA methylation status could be influenced by a wide variety of confounding factors consisting of lifestyle habits, parental smoking, body mass index, ethnicity, socioeconomic status, and environmental exposures. Fourthly, due to inadequate data on clinical indicators in the healthy controls, the determination of the relationship between Alu methylation levels and biochemical indicators was unachievable. Finally, the differences concerning the studied exposures from the selection bias were inevitable, since the subjects in this study were based on hospital participants, which were rather than on the general population.

5. Conclusion

This study was the first to demonstrate that Alu hypomethylation could be a useful biomarker for monitoring the early progression of ATDILI in TB patients. Supporting this, TB patients with ATDILI demonstrated significantly lower Alu methylation levels than those without ATDILI and age-and gender-matched healthy controls. Correspondingly, aberrant Alu methylation was independently associated with raised levels of liver enzyme indicators including AST and ALT. Future investigations on both gene-specific methylation and the molecular mechanisms underlying retrotransposon hypomethylation will help to

elucidate the significant involvement of Alu hypomethylation in ATDILI, which may help decrease the manifestation of liver injury in TB patients and its negative outcomes.

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