Original article

Prevalence of *NUDT15c.415C>T* and *ITPAc.94C>A* among Thai pediatric patients with acute lymphoblastic leukemia using simultaneous multiplex ARMS PCR

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Abstract:

Introduction: *Nucleoside diphosphate-linked moiety X-type motif 15 c.415C>T (NUDT15c.415C>T) and inosine triphosphate pyrophosphatase c.94C>A (ITPAc.94C>A) are associated with a decreased degradation of 6-mercaptopurine (6-MP) among patients with acute lymphoblastic leukemia (ALL), resulting in a risk of bone marrow cytotoxicity (myelotoxicity).* ***Objective:*** *The study aimed to investigate the prevalence of NUDT15c.415C>T and ITPAc.94C>A genes among pediatric patients with ALL at Siriraj Hospital for surveillance of 6-MP-related side effects.* ***Material and Methods:*** *Sample specimens were collected from 222 pediatric patients and analyzed using a multiplex ARMS PCR method that was developed to detect the polymorphism of NUDT15c.415C>T and ITPAc.94C>A in a single detection.* ***Results:*** *The proportions of homozygous NUDT15c.415C>T and ITPAc.94C>A were 0.90 and 4.50%, respectively, while the proportions of heterozygous NUDT15c.415C>T and ITPAc.94C>A were 13.10 and 29.70%, respectively. The proportions of those harboring both the NUDT15c.415C>T and ITPAc.94C>A alleles were 5.40, and 0.90% of all patients harboring the heterozygous genetic pattern of one gene and homozygous for another gene. The proportion of those with wild-type genotypes of both genes was 45.50%. Co-occurrence of the homozygous genotype in both genes was not found in this cohort. In addition, the allele frequencies of NUDT15c.415C>T and ITPAc.94C>A were 10.81, and 22.70% respectively.* ***Conclusion:*** *The genetic polymorphism of ITPAc.94C>A appeared to be more prevalent than that of NUDT15c.415C>T among Thai pediatric patients with ALL. Interestingly, in this case, the heterozygous genetic pattern of one gene was related to the homozygous of another gene. Further studies are warranted to elucidate the clinical significance of ITPAc.94C>A on myelotoxicity among Thai pediatric patients treated with thiopurine. Furthermore, this method offers time-efficient and economic advantages as it can evaluate both polymorphism in a single procedure.*

Keywords : ● *ITPAc.94C>A* ● *NUDT15c.415C>T* ● *Acute lymphoblastic leukemia* ● *6-mercaptopurine* ● *Multiplex ARMS PCR*

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นิพนธ์ต้นฉบับ

การศึกษาความชุกของยีน *NUDT15c.415C>T* และยีน *ITPAc.94C>A* ในผู้ป่วยเด็กไทยโรคมะเร็งเม็ดเลือดขาวเฉียบพลันชนิดลิมโฟบลาสติก ด้วยเทคนิค multiplex ARMS PCR

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บทคัดย่อ

บทนำ *ยีน Nucleoside diphosphate-linked moiety X-type motif 15 ชนิด c.415C>T (NUDT15c.415C>T) และยีน Inosine triphosphate pyrophosphatase ชนิด c.94C>A (ITPAc.94C>A) มีความสัมพันธ์ต่อการสลายตัวของระดับยา 6-mercaptopurine (6-MP) ในผู้ป่วยโรคมะเร็งเม็ดเลือดขาวเฉียบพลันชนิดลิมโฟบลาสติก (Acute lymphoblastic leukemia; ALL) ซึ่งส่งผลให้เกิดความเสี่ยงต่อภาวะเป็นพิษต่อเซลล์เม็ดเลือดในไขกระดูก (myelotoxicity) ในกลุ่มผู้ป่วยดังกล่าว* ***วัตถุประสงค์*** *เพื่อศึกษาความชุกของยีน NUDT15c.415C>T และ ITPAc.94C>A ในผู้ป่วยเด็กโรค ALL ในโรงพยาบาลศิริราช ทำให้เพิ่มการเฝ้าระวังผลข้างเคียงจากยา 6-MP* ***วัสดุและวิธีการ*** *รวบรวมและวิเคราะห์ผลการตรวจยีนดังกล่าวในกลุ่มตัวอย่างผู้ป่วยเด็กโรค ALL จำนวน 222 ราย ด้วยเทคนิค multiplex ARMS PCR ซึ่งเป็นวิธีที่พัฒนาเพื่อตรวจทั้งสองชนิดพร้อมกัน* ***ผลการศึกษา*** *จากการศึกษารูปแบบพันธุกรรมหรือจีโนไทป์ในกลุ่มตัวอย่างดังกล่าว พบยีน NUDT15c.415C>T และยีน ITPAc.94C>A มีรูปแบบพันธุกรรมโฮโมไซกัสเท่ากับร้อยละ 0.90 และ 4.50 ตามลำดับ รูปแบบพันธุกรรมเฮเทอโรไซกัสเท่ากับร้อยละ 13.10 และ 29.70 ตามลำดับ รูปแบบพันธุกรรมเฮเทอโรไ ซกัสร่วมกันระหว่างยีนทั้งสองชนิดร้อยละ 5.40 และรูปแบบพันธุกรรมเฮเทอโรไซกัสของยีนใดยีนหนึ่งร่วมกับโฮโมไซกัสของอีกยีนหนึ่งพบร้อยละ 0.90 รูปแบบพันธุกรรมปกติตรวจพบร้อยละ 45.50 ในขณะที่ไม่พบรูปแบบพันธุกรรมโฮโมไซกัสร่วมกันระหว่างยีนทั้งสองชนิดในกลุ่มตัวอย่าง ความถี่ของอัลลีลผิดปกติของยีน NUDT15c.415C>T และ ITPAc.94C>A ตรวจพบร้อยละ 10.81และ 22.70 ตามลำดับ* ***สรุป*** *Genetic polymorphism ของยีน ITPAc.94C>A พบได้บ่อยกว่ายีน NUDT15c.415C>T ในผู้ป่วยเด็กไทยโรค ALL และน่าสนใจในกรณีพบรูปแบบพันธุกรรมเฮเทอโรไซกัสของยีนใดยีนหนึ่งร่วมกับโฮโมไซกัสของอีกยีนหนึ่ง จึงควรทำการศึกษาเพิ่มเติมเพื่อดูความสัมพันธ์ทางคลินิกของยีน ITPAc.94C>A ที่อาจส่งผลให้เกิดความเสี่ยงในการเกิดภาวะเป็นพิษต่อเซลล์เม็ดเลือดในไขกระดูกผู้ป่วยที่ได้รับยากลุ่ม thiopurine นอกจากนี้วิธีการที่ใช้สามารถประหยัดเวลารวมถึงค่าใช้จ่าย และยังตรวจหาความหลากหลายของทั้งสองยีนได้ในครั้งเดียว*

คำสำคัญ : ● *ยีน ITPAc.94C>A* ● *ยีน NUDT15c.415C>T* ● *โรคมะเร็งเม็ดเลือดขาวเฉียบพลันชนิดลิมโฟบลาสติก* ● *6-mercaptopurine* ● *Multiplex ARMS PCR*

วารสารโลหิตวิทยาและเวชศาสตร์บริการโลหิต. 2566;33:199-206.

Introduction

Acute lymphoblastic leukemia (ALL) represents the most common type of pediatric leukemia, accounting for approximately 80% of all cases[1]. The interval of treatment lasts approximately three years, and during the maintenance phase, 6-mercaptopurine (6-MP) and methotrexate (MTX) are the mainstay therapy. However, some patients may experience severe toxicity such as myelotoxicity and hepatotoxicity[2], which may result in treatment interruption and ultimately increase the risk of disease relapse. Polymorphism of the genes involved in thiopurine metabolism including thiopurine methyltransferase (*TPMT*), nucleoside diphosphate-linked moiety X-type motif 15 (*NUDT15*) and inosine triphosphatase (*ITPA*) can lead to variable degradation of the toxic metabolites of thiopurine. Such polymorphism can vary among ethnicities[3], for instance, *TPMT* is prevalent among Caucasians[4,5], whereas *ITPA* and *NUDT15* are prevalent among Asians[6]. In addition, *NUDT15* polymorphism is uncommon in European populations except for the Spanish population, and is even rarer in African populations[7,8]. Regarding clinical implications, the prevailing guidelines advocate reducing the dosage of thiopurine based on the presence of *TPMT* and *NUDT 15* polymorphisms, while a unanimous consensus is yet to be reached concerning the *ITPA* polymorphism[9].

Several methods including polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), allele-specific polymerase chain reaction (AS-PCR), pyrosequencing and multiplex high-resolution melting analysis (HRMA) and multiplex amplification refractory mutation system polymerase chain reaction (multiplex ARMS PCR), have been used to detect the *ITPA* and *NUDT15* polymorphisms[10-13]. The objective of the present research was to study the genetic frequencies of *NUDT15c.415C>T* and *ITPAc.94C>A* among Thai pediatric patients with ALL using a multiplex ARMS PCR method to detect the polymorphism of *NUDT15c.415C>T* and *ITPAc.94C>A* in a single detection, which could offer time and cost savings.

Subjects and methods

In total, 222 leftover blood specimens from Thai pediatric patients receiving a diagnosis with ALL were obtained from the Hematology and Oncology Division, Department of Pediatrics, Siriraj Hospital, Mahidol University from January 2015 to December 2021. This study was approved by the Siriraj Institutional Review Board, reference number: COA No. Si 787/2021. All the specimens were used for genomic DNA (gDNA) extraction using the salting-out method[14]. Genotypes of *NUDT15c.415C>T* and *ITPAc.94C>A* were detected from gDNA samples (200-300 ng) by specific primers (Table 1)[15,16], for which type-different concentrations of specific primers were simultaneously used to detect the wild-type (WT) and mutant (MT) alleles in PCR buffer containing 1.5 mM $MgCl_2$, 200 µM dNTPs, 1x Q-solution and 1.25 U HotStar *Taq* DNA polymerase (Qiagen, Hilder, Germany). The PCR reaction started from the predenaturation at 95°C for 15 min followed by 30-cycle amplification including denaturation at 94°C for 1 min, annealing at 65°C for 45 sec and then extension at 72°C for 1 min. The last step was the final extension at 72°C for 10 min. Both PCR products of the WT and MT alleles were parallel loaded and compared with a 100 bp marker, as shown in Figure 1, using 1.5% agarose gel electrophoresis with a constant 100 V for 35 min followed by ethidium bromide staining and UV visualization.

Results

The 222 pediatric subjects comprised 126 males (56.80%) and 96 females (43.20%), and the median age at diagnosis was five years (range 3 months to 18 years). Among the included patients, 194 (87.4%) received a diagnosis as pre-B ALL, while 28 (12.60%) received a diagnosis as T-cell ALL. The prevalences of genetic polymorphisms of *NUDT15c.415C>T* and *ITPAc.94C>A* reported as homozygous *NUDT15c.415C>T and ITPAc.94C>A* were 0.90 and 4.50% respectively. whereas the values for heterozygous *NUDT15c.415C>T* and *ITPAc.94C>A* were 13.10 and 29.70% respectively. The proportion of

Table 1 Designed specific primers for *ITPAc.94C>A* and *NUDT15c.415C>T* used in the multiplex ARMS PCR

Common name	Primer sequence 5'->3'	Amplicon size (bp)	Primer concentration (μM)	Reference
ITPAc.94C>A	Reverse: TTC CAC GAA CAT GTG TGA ATG CAG C Forward: (WT): CGT TCA GAT TCT AGG AGA TAA GTT CC Forward: (MT): CGT TCA GAT TCT AGG AGA TAA GTT CA	533	0.2	(15)
NUDT15c.415C>T	Reverse: GCT GAA AGA GTG GGG GAT AC Forward: (WT): GGA GCT TTT CTG GGG ACT GC Forward: (MT): GGA GCT TTT CTG GGG ACT GCT	259	0.2	(16)
Internal control for multiplex ARMS PCR	Forward: GCT TAG CAC AAG CAG AGA CCT GAC G Reverse: TTC CAC GAA CAT GTG TGA ATG CAG C	765	0.1	(15)

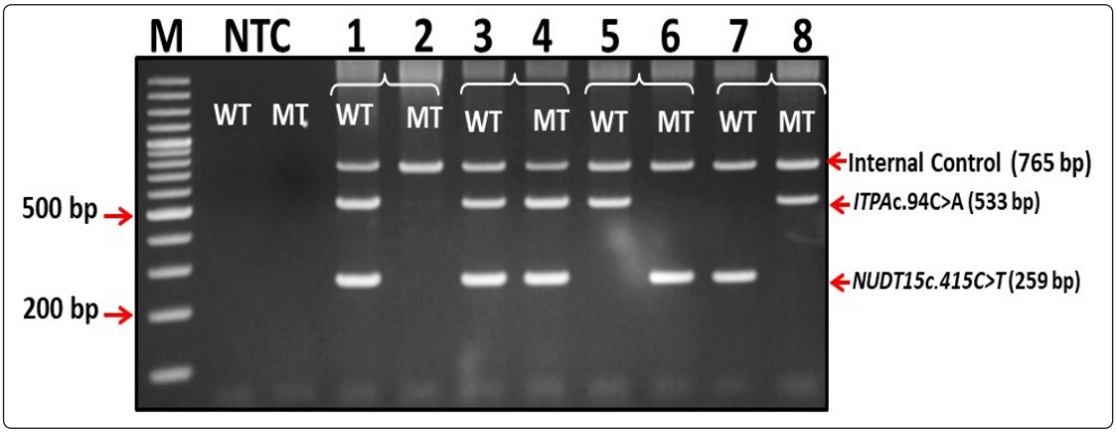

Figure 1 PCR products of the wild-type (WT) allele and mutant (MT) alleles detected for *NUDT15c.415C>T* and *ITPAc.94C>A* genotypes using multiplex ARMS-PCR by 1.5% agarose gel electrophoresis: M, 100 bp marker; NTC, non-template; WT, detected wild-type allele; MT, detected mutant allele; Lanes 1, 2 wild-types of *NUDT15c.415C>T* and *ITPAc.94C>A*; Lanes 3, 4, both heterozygous *NUDT15c.415C>T* and *ITPAc.94C>A*; Lanes 5, 6, homozygous *NUDT15c.415C>T*; Lanes 7, 8, homozygous *ITPAc.94C>A*. The 765 bp amplified products served as the internal control. The 533 bp and 259 bp amplified products were the *ITPAc.94C>A* and *NUDT15c.415C>T* specific products, respectively.

Table 2. Prevalence of *NUDT15c.415C>T* and *ITPAc.94C>A* among 222 Thai pediatric patients receiving a diagnosis of acute lymphoblastic leukemia

Genotype	Number (%)
ITPAc.94C>A genotype frequency	
- CC	101 (45.50)
- CA	66 (29.70)
- AA	10 (4.50)
NUDT15c.415C>T genotype frequency	
- CC	101 (45.50)
- CT	29 (13.10)
- TT	2 (0.90)
Both *ITPAc.94C>A* and *NUDT15c.415C>T* genotype frequency	14 (6.30)
- CA/CT	12 (5.40)
- AA/CT	1 (0.45)
- CA/TT	1 (0.45)

ITPA: inosine triphosphate pyrophosphatase; *NUDT15*: nucleoside diphosphate-linked moiety X-type motif 15

those carrying both heterozygous *NUDT15c.415C>T* and *ITPAc.94C>A* alleles was 5.40%, while the proportion of those with the wild-type genotypes was 45.5%. In this cohort, 0.90% of all patients harbored the heterozygous genetic pattern of one gene and homozygous of another gene. Details of the prevalence of the genetic polymorphism of both genes are shown in Table 2. Also, the allele frequencies of the *NUDT15c.415C>T* and *ITPAc.94C>A* genes were 10.81 and 22.70%, respectively. The time required for performing the laboratory test using simultaneous multiplex ARMS PCR was three hours, whereas it took six hours using AS-PCR. The unit cost for simultaneous multiplex ARMS PCR was 2020 THB, whereas 4040 THB for AS-PCR.

Discussion

Personalized medicine using the genetic information of patients to provide appropriate treatment for individuals appears to be an increasing trend among physicians[17]. Likewise, recognizing patients harboring the *TPMT* polymorphism, the first identified genetic polymorphism of purine metabolism, is important, as they could experience severe toxicities with the traditional thiopurine treatment, which could lead to the need for treatment interruption, which could ultimately affect the survival chances of such patients[18]. This finding highlights the role of individualized medicine for those treated with thiopurine drugs, especially patients with ALL. Table 3 illustrates the clinical significance of *NUDT15* and *ITPA* polymorphism among Thai patients[19-24].

In this study, we found that the frequency of *ITPAc.94C>A* was more prevalent than that of *NUDT15c.415C>T*. Related studies reported two common genetic polymorphisms of *ITPA*: *ITPAc.94C>A*, the most common polymorphism and IVS2+21A>C, the second-most common polymorphism. The enzymatic activity in homozygous *ITPA* is less than 1%, whereas that in heterozygous is approximately 20%[25]. Decreased enzymatic activity has been reported to be associated with a risk of neutropenia[26,27] and transaminitis[28] in those treated with a thiopurine. The frequency of the *ITPAc.94C>A* allele in Asian populations has been reported to be 30, 15.5, 16, 18.1 and 11% in Korean, Japanese, Malaysian, Chinese and Indian populations, respectively[15,29-31].

NUDT15c.415C>T was first reported to be associated with toxicity in Korean patients treated with a thiopurine[32]. The allele frequency of this in Japanese, Chinese and Thai populations was reported to be 16, 11.6 and 17%, respectively[22,33,34], while the frequency was reported to be less than 1% among Caucasians[35]. The

Table 3 Clinical significance of *NUDT15* and *ITPA* polymorphism among Thai patients treated with 6-MP.

Polymorphism	Clinical significance
ITPA and NUDT15 [19]	Heterozygous and homozygous polymorphism required dose reduction during maintenance therapy.
NUDT15 [20]	Heterozygous and homozygous polymorphism had higher incidence of neutropenia during the first 3 months of maintenance and required dose reduction.
NUDT15 [21]	Heterozygous and homozygous polymorphism required dose reduction of 26.8 and 75% respectively during the first 6 months of maintenance therapy.
ITPA and *NUDT15* [22]	Only *NUDT15* polymorphism was associated with neutropenia.
ITPA and *NUDT15* [23]	Only *NUDT15* polymorphism was associated with neutropenia and dose reduction.
ITPA [24]	Homozygous *ITPA* was associated with dose reduction and transaminitis.

6-MP: 6-mercaptopurine; *ITPA*: inosine triphosphate pyrophosphatase; *NUDT15*: nucleoside diphosphate-linked moiety X-type motif 15.

homozygous or heterozygous *NUDT15* reportedly caused increased hematologic toxicity in two related studies[20,36]. Likewise, the prevalences of *NUDT15c.415C>T* and *ITPAc.94C>A* in this study are in line with the aforementioned studies in Thai populations. In addition, the multiplex AMRS PCR method can be used to simultaneously detect both *NUDT15c.415C>T* and *ITPAc.94C>A* by their specific detecting primers. This method offers time-efficient and economic advantages. These results may assist physicians in providing appropriate treatment for those treated with thiopurine because the guidelines of adjusting the thiopurine dose among patients with *NUDT15* polymorphisms has already been established[9].

In summary, the genetic polymorphism of *ITPAc.94C>A* appears to be more prevalent than that of *NUDT15c.415C>T* in Thai pediatric patients with ALL. Further studies are warranted to elucidate the clinical significance of *ITPAc.94C>A* on myelotoxicity among Thai pediatric patients treated with a thiopurine.

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