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Research Article

Prevalence of NUDT15C.415C>T Among Filipino Children Diagnosed with Acute Lymphoblastic Leukemia

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Abstract:

Background: Purine antagonists like 6-mercaptopurine (6-MP) are widely used treatment agents for acute lymphoblastic leukemia (ALL). However, treatment interruptions due to 6-MP toxicity continue to be a commonly encountered problem. Recently, polymorphisms involving the nudix hydrolase (*NUDT15)* gene have been discovered as a major genetic cause for 6-MP related toxicity. Unfortunately, there is lack of study on *NUDT15* variants in the Philippines.

Objectives: This study aimed to determine specifically the prevalence of the *NUDT15c.415C>T* among Filipino children diagnosed with ALL. It also aimed to investigate possible association with 6-MP myelosuppression.

Methods: This was a cross sectional study which included Filipino subjects <19 years old diagnosed with ALL. Peripheral blood was used and from these genomic DNA was extracted. The *NUDT15c.415C>T* gene was amplified by real-time PCR and analyzed by high-resolution melting (HRM) to detect variants.

Results: A total of 89 subjects were included and from these 18 (20.2%) were found positive for the *NUDT15c.415C>T*. In terms of blood count, *NUDT15c.415C>T* positive subjects have significantly lower hemoglobin, white blood cell, neutrophil, absolute neutrophil count (ANC), and platelet. Regression analysis showed that the *NUDT15 c.415C>T* was associated with lower hemoglobin and ANC.

Discussion and Conclusion: This study identified high prevalence rate of *NUDT15 c.415C>T* among the Filipino subjects. This study also identified that it was associated with significant myelosuppression. This study emphasizes the need to pursue pharmacogenetic analysis among Filipinos to identify *NUDT15* to avoid unnecessary interruptions by treatment related myelosuppression.

Keywords: Acute lymphoblastic leukemia; Filipino; 6-mercaptopurine; *NUDT15c.415C>T*; myelosuppression

Introduction

Acute lymphoblastic leukemia (ALL) is the most common cancer in children, adolescents and young adults younger than 20 years, accounting for 18.8 percent of all cancer cases in this age group.[1] According to the Leukemia & Lymphoma Society, the 5-year survival rate of ALL is 91.9% for children and adolescents younger than 15 years, and 94.1 percent for children younger than 5 years.[2] The disease-free survival has greatly

increased with the improved treatment regimens. Despite this, treatment interruptions due to myelosuppression is a common adverse event and results in a higher relapse rate, making ALL the second leading cause of cancer death among children.[3]

6-Mercaptopurine (6-MP) is an important anticancer agent and remains a backbone of most ALL regimens.[4] It is commonly used as a part of the consolidation, intensification and maintenance phases of the treatment. However, it is not uncommon to encounter severe myelosuppression and agranulocytosis while on 6-MP therapy.[5] This is partly due to the large interindividual and intraindividual variations in 6-MP bioavailability and cellular pharmacokinetics affecting patient dosing. Patients receiving identical doses per body surface area may experience varied systemic and intracellular drug toxicity.[1] As a result, different strategies have been employed to address this challenge with most attempts resulting to varied outcome. To date, the utilization of genetic polymorphisms and pharmacogenomics that determine 6-MP efficacy and toxicity have been employed to improve ALL treatment.[6]

Recently, single nucleotide polymorphisms (SNPs) involving the Nudix hydrolase 15 (*NUDT15*) gene have been identified as a major genetic cause for 6-MP related bone marrow suppression.[7] This gene encodes an enzyme that belongs to the Nudix hydrolase superfamily which is a negative regulator of thiopurine activation and toxicity. Members of this superfamily catalyze the hydrolysis of nucleoside diphosphates, including substrates like 8-oxo-dGTP, which are a result of oxidative damage, and can induce base mispairing during DNA replication, causing transversions. The gene is in the long arm of chromosome 13 at position 14.2 and mutations in this gene result in poor metabolism of 6-MP.[22]

There are four coding variants for *NUDT15* currently identified: *NUDT15*3* (rs116855232, c.415C > T), *NUDT15*5* (rs186364861, c.52G > A), *NUDT15*6* (rs869320766, c.36_37insGGA GTC), and *NUDT15*7* (rs766023281, c.101G > C). The *NUDT15 c.415C>T* is more common in East Asians.

A meta-analysis by Yin et al. (2017) demonstrates that *NUDT15* results to thiopurine intolerance dose.[23] This results to patients with severe neutropenia to have higher risk of severe infections, causing interruption of scheduled treatment, which is associated with unfavorable outcome and prognosis.[24] Studies identified that truncated chemotherapy may result to 5-year event free survival as low as 60% even for non–high-risk ALL patients.[23] In addition, studies have shown that recurrent unwarranted treatment interruptions as an adverse factor for increased risk of second malignancies.[26]

By doing this study, we contribute to current knowledge regarding the presence of *NUDT15* genetic polymorphisms in Asia, since there are no studies in the Philippines on Filipino children on this. We can also support future recommendations to do preemptive *NUDT15* genotyping in patients with ALL prior to initiating therapy. This study will establish the role of *NUDT15* in thiopurine metabolism in Filipino children with ALL, and will investigate the possible correlation of genetic polymorphisms to dose-related toxicity. Moreover, this research will help future researches that will investigate dosing strategies and formulate guidelines for dose-adjustments in Filipino children.

Objectives

The objective of this study is to determine the specifically prevalence of the *NUDT15c.415C>T* among Filipinos and to identify whether this is associated with significant myelosuppression.

Methods

Study Design and Setting

This is a single center ambispective longitudinal cross-sectional study which included Filipino children <19 years old, diagnosed with ALL from January 2012 to January 2022.

Inclusion Criteria and Exclusion Criteria for Subject Selection

The study only included include patients who are currently undergoing chemotherapy and is in the maintenance phase of chemotherapy, patients who are off-therapy but are on regular clinic follow-up, and are treated using the Modified ALL-BFM (Berlin-Frankfurt-Munster) Protocol. The patient should also fulfill the following criteria prior to the start of the maintenance phase: absolute neutrophil count (ANC) 750/mm^3, platelet count 75,000/mm^3, serum creatinine ≤2 x the UNL, alanine aminotransferase (ALT) ≤ 3 UNL, bilirubin ≤1.5 x UNL, and no active systemic infection or evidence of hepatic SOS. The study excluded patients with concomitant liver disease that is not related to chemotherapy and those managed using protocols other than the Modified ALL-BFM (Berlin-Frankfurt-Munster) Protocol.

Description of Study Procedure

Informed consent form was discussed by the investigator to the patient and the parents. The patients were then screened for eligibility using the inclusion and exclusion criteria. Once the parent agrees and signs the informed consent and assent forms, eligible patients were enrolled.

The CBC were taken after the first two weeks of the first cycle of maintenance phase of chemotherapy of the Modified BFM Protocol. For patients who have completed their treatment, review of electronic medical records from the online system was done to obtain the results of the laboratory tests.

For this study, using the Modified BFM Protocol, the goal is to achieve the dose of 6-MP at 75mg/m^2 daily. However, doses were started at 50mg/m^2 then titrated according to the degree of myelosuppression with a target ANC of 750/mm^3 and platelet count of 75,000/mm^3.

NUDT15 c.415C>T Determination

Peripheral blood extraction for the *NUDT15 c.415C>T* determination was done together with the patient's regular blood work extraction anytime during the course of treatment. Those who are off treatment, peripheral blood extraction was done during regular clinic follow ups together with the routine CBC. A total of

8 milliliters (mL) of peripheral blood was collected and placed in 2 EDTA tubes and underwent buffy coat separation by centrifugation at 3000 rpm, for 15 minutes at room temperature (25ºC). The buffy coat layer was then be collected and placed in 1.5mL conical tubes. These then underwent microcentrifugation at the same speed, duration, and temperature. Excess layer of plasma and RBC were be removed. Samples were stored at -80 ºC, at the Research and Biotechnology Division prior to processing.

Genomic DNA extraction process was performed on peripheral blood in EDTA-tubes using the QIAamp DNA Blood Kit Mini Handbook (51104, Qiagen). The total genomic DNA concentration was determined using the Nanodrop v1000 spectrophotometer (ThermoFisher Scientific). The *NUDT15 c.415C>T* was tested using two primer sets (Table 1).

The Step-down PCR protocol using Real-time PCR is described in Table 2. The following reagents and concentrations composed the Real Time PCR by High Resolution Melt (HRM) Analysis: 0.7µL (0.7µM) of each of the primers, 5.0µL of Qiagen Type-It HRM PCR Master Mix, 2.0µL Template DNA (50ng), and Nuclease-free water added up to a final volume of 10µL.

Description of Outcome Measures

The primary outcome for this study is to determine the prevalence of *NUDT15 c.415C>T* variant in Filipino patients with ALL who are taking 6-MP. The secondary outcome is to determine whether *NUDT15 c.415C>T* is correlated with development with myelosuppression upon the start of the maintenance therapy where 6-MP was initiated. Significant myelosuppression is defined as values less than ANC of 750/mm^3 and platelet count of 75,000/mm^3. Comparison between with *NUDT15 c.415C>T* variant positive versus negative was done to identify whether the risk for toxicity is higher compared to the other.

Table 1: Primer sets used in testing for the *NUDT15 c.415C>T* variant.

Primer	Sequence	Procedure
PCP 31 (For)	5′-GACCAGCTTTTCTGGGGACT-3′	High Resolution Melt
PCP 32 (Rev)	5′-TCCCACCAGATGGTTCAGAT-3′	High Resolution Melt

Table 2: Step-down PCR protocol using Real-time PCR.

Stage	Step	Temperature	Time other details
Holding	Enzyme Activation	50^{0}C	2 mins
		95^{0}C	10 mins
Cycling (40 cycles)	Denaturation	95^{0}C	15 secs
	Anneal/Extend	55 ^{0}C	1 min
Melt Curve/ Dissociation	Denature	95^{0}C	15 secs
	Anneal	60^{0}C	1 min
	High Resolution Melting Collection Mode: Continuous	95^{0}C	1 sec

Sample Size Computation

To estimate the sample size, Cochran's formula was used. Where the margin of error (e) is set at ±5% (95% confidence). In a study done by, Liang et al[6], they found out that the risk allele frequency of *NUDT15* in Taiwanese children is at 11.6% hence p = 0.11. Using this formula, the minimum computed sample size is 81.

Data Analysis

Descriptive statistics using frequency distribution and measures of central tendencies were used on the subjects and stratified per exposure group. Difference in means for quantitative variables per exposure group was performed using independent t-test. Difference in proportion for qualitative variables per exposure group was performed using Chi-square test. Association between presence of *NUDT15 c.415C>T* variants and toxicity parameters was performed using regression analysis. All tests were two-tailed and considered significant at p<0.05.

Ethical Considerations

Institutional Ethics Review Board (IERB) approval from the St. Luke's Medical Center – Quezon City was obtained prior to the initiation of the study.

Conflict of Interest

The authors declare no conflict of interest.

Results

A total of 89 subjects were included the study (Table 3). Out of the 89 subjects, 18 (20.2%) was positive for the *NUDT15 c.415C>T* variant tested.

In terms of the blood count (Table 4), *NUDT15 c.415C>T* positive subjects have significantly lower hemoglobin (9.3g/dl, p-value<0.001), white blood cell count (2158mm^3, p-value<0.001), neutrophil count (28, p-value<0.001), ANC (594/mm^3, p-value<0.001) and platelet count (167,338/mm^3, p-value<0.001).

Table 4: Comparison between NUDT15 c.415C>T positive and negative in terms of blood cell To estimate the sample size, Cochran's formula was used. Where count.

	NUDT15 c.415C>T Positive	NUDT15 c.415C>T Negative	p-value
Hemoglobin (NV = 13 - 17g/dl)	9.3 ± 0.99	11.0 ± 1.70	0.001*
WBC (NV = 4,800 - 10,800 mm^3)	2,158 ± 683.5	3,430 ± 1,825.3	0.001*
Neutrophil (NV = 40 - 74)	28 ± 17	48 ± 22	0.001*
Platelet count (NV = 150,000 - 400,000/mm^3)	167,338 ± 106,490	265,450 ± 115,925	0.002*
ANC (NV ≥ 1,500/mm^3)	594 ± 416	1,800 ± 1401	0.001*

NV = Normal Value

*p-value less than 0.001

Running the regression analysis, *NUDT15 c.415C>T* positive variant was identified to be associated with lower hemoglobin (p-value<0.005) and lower ANC (p-value<0.010).

Discussion

It was first reported by Yang et al. in 2014 the NUDT15 variant conferring susceptibility to thiopurine-induced toxicity.[9] Since then, there have been numerous publications that further supported Yang et al.'s findings. These studies identified that this variant is most common in East Asians, rare in Europeans, and not observed in Africans.[10] They have identified ancestry-associated differences. Though studies have been conducted on other Asian countries, there is still lack of data regarding *NUDT15* polymorphisms in the Filipino population. Additionally, there is lack of published studies involving other SNPs like thiopurine methyltransferase (*TPMT*) and inosine triphosphate pyrophosphatase (*ITPA*) in the Philippines. Factors, especially our multiracial ethnicity, may contribute to ancestry associated differences among these SNPs.

The results of this study have identified 20.2% prevalence of the *NUDT15 c.415C>T*. This is higher in contrast with the study by Jena et al. (2021) where pooled prevalence rate of 16.5% among South Asians.[11] On the other hand the results of the study are comparable to East Asian populations, including those in Chinese (24.5%), Japanese (20.0%), and Korean (24.0%).[12-14] However, among other Southeast Asian countries, it is lower compared to Vietnam (32.9%) but higher than Thailand (15.0%).[15-16]

This study identified *NUDT15 c.415C>T* positive variants was associated with myelosuppression during the maintenance phase of the chemotherapy treatment, specifically significantly lower hemoglobin and lower ANC. Findings of neutropenia and low ANC has been previously documented in various studies primarily as a result of 6-MP induced myelotoxicity like that of Tanaka et al. (2015), Schaeffeler et al. (2019) and Wang et al. (2019).[17-19]

On the other hand, in a study by Zhu et al. (2018), one ALL patient positive for *NUDT15 c.415C>T* variant experienced severe myelosuppression resulting to pancytopenia with hemoglobin of 65g/L, WBC of $1x10^9$/L, ANC of $0.01x10^9$/L and platelet count of $19x10^9$/L after two weeks of standard 6-MP treatment in the consolidation phase.[20] Involvement of other cell lines therefore is not uncommon finding in *NUDT15* variant associated 6-MP induced myelosuppression. This could also explain the lower hemoglobin count observed in this study.

Conclusion

This study identified the prevalence rate of *NUDT15 c.415C>T* positive variant at 20.2% which is comparable to East Asian countries. This study also identified correlation between the NUDT15 c.415C>T positive variant with myelosuppression, specifically lower hemoglobin count and ANC.

This is the first study that identified the prevalence of *NUDT15 c.415C>T* variant in the Filipino population. The impact of *NUDT15* polymorphisms on the management of ALL, specifically on myelosuppression, is emphasized. This study also emphasizes the need to be recognize potential 6-MP associated toxicity, the need to pursue pharmacogenetic analysis to identify polymorphisms that may affect 6-MP administration and the need to avoid unnecessary interruptions that may negatively affect overall survival.

Limitations and Recommendations

The study only utilized HRM and is limited for detection of the variant of interest. As a limitation, the subjects were not further identified to either being homozygous or heterozygous. We recommend Sanger sequencing be performed.

We also recommend that future studies include the Kaplan-Meier to analyze the overall survival of subjects included in the study.

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