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ORIGINAL ARTICLE

NUDT15 gene polymorphism related to mercaptopurine intolerance in Taiwan Chinese children with acute lymphoblastic leukemia

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A recent study identified a variant of the *NUDT15* gene (rs116855232 C>T) associated with intolerance to thiopurine in Korean patients with Crohn's disease. This study prompted us to substantiate the finding in a Taiwanese population. Four hundred and four children with acute lymphoblastic leukemia (ALL), and 100 adults with chronic immune thrombocytopenic purpura or localized lymphoma having normal bone marrow were examined. Two candidate gene approaches, pyrosequencing for *NUDT15* and TaqMan assay for thiopurine methyltransferase (*TPMT*) genotyping (rs1142345 A>G), were performed. We showed a risk allele frequency of *NUDT15* of 11.6% in children with ALL and 15.5% in adults. By contrast, the risk allele frequency of *TPMT* was only 1.6% in children with ALL and 0.5% in adults. The high frequency of risk variant for *NUDT15,* but not the very low frequency of risk variant for *TPMT,* was closely associated with the intolerance to mercaptopurine in children with ALL in Taiwan, contrast to that of European descent. In regard to *NUDT15* polymorphism, the maximal tolerable daily doses of mercaptopurine in homozygotes, heterozygotes and wild-type groups were 9.4 mg m^{-2}, 30.7 mg m^{-2} and 44.1 mg m^{-2}, respectively. The outcomes did not differ significantly among the different genotypes.

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INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most prevalent pediatric cancer with cure rates approaching 90% with contemporary treatment.[1–5] For reasons that are still poorly understood, patients with ALL require long-term continuation therapy. The combination of weekly methotrexate and nightly mercaptopurine constitutes the standard 'backbone' of ALL continuation regimens. The accumulation of higher intracellular levels of thioguanine nucleotides, active metabolites of mercaptopurine and tailoring of mercaptopurine doses to the limits of tolerance have been associated with a better treatment outcome.[6,7] However, it is counterproductive to use methotrexate and mercaptopurine overzealously because excessive myelosuppression can preclude further chemotherapy, leading to reduced overall dose intensity.[8]

Thiopurine methyltransferase (*TPMT*) is a major catabolic enzyme of mercaptopurine.[9] Genetic polymorphisms in *TPMT* greatly affect the enzymatic activity of the protein product. In the Western countries, ~10% of the people of European descent inherit one non-functional variant of *TPMT* with heterozygous deficiency of the enzyme, and 1 in 300 have two non-functional variants with homozygous deficiency.[10,11] Compared with the 90% of patients with wild-type enzyme activity who can tolerate mercaptopurine at dosage of 75 mg m^{-2} per day, a large proportion of heterozygous patients require dose reduction and patients of homozygotes even need a 10-fold dose reduction to avoid excessive hematopoietic toxicity.[12]

It has been well recognized that patients in the Far East have poorer tolerance to mercaptopurine as compared with those in the West. However, previous studies failed to yield an explanation. Actually, the prevalence of *TPMT* heterozygotes and homozygotes is rare in Far East.[13,14] Recently, a study has identified a variant of the *NUDT15* gene (rs116855232 C>T) associated with intolerance to thioguanine in Korean patients with Crohn's disease.[15] This intriguing study prompted us to use candidate gene approach to substantiate the finding in a Taiwan population.

MATERIALS AND METHODS

Patients and samples

Four hundred and four children aged < 18 years at diagnosis of ALL, who were treated at Chang Gung Memorial Hospital at Linkou and Mackay Children's Hospital in Taiwan between 1996 and 2014, had achieved complete remission and had available samples, were enrolled in this study with the written informed consent from the parents and/or the patients as appropriate. These patients were enrolled in the Taiwan Pediatric Oncology Group 97, 2002 and 2013 protocols. Details of the treatment have been reported previously.[16,17] Here we described only the maintenance therapy with methotrexate and mercaptopurine, which were started at 40 mg m^{-2} per week and 60 mg m^{-2} per night, respectively. Dosages were titrated to keep WBC count between 1800 and 3000 mm^{-3}, absolute neutrophil count between 500 and 1200 mm^{-3} (with the exception of the count 1 week after dexamethasone treatment), and platelet count ⩾ 50000 mm^{-3}. If counts were low, mercaptopurine was the first drug to be reduced in dosage by 25% decrements. When

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Table 1. Clinical characteristics of children with acute lymphoblastic leukemia according to *NUDT15* genotypes

Feature	*NUDT15*		
	CC	*TC*	*TT*
Total number	315	84	5
Age (years) (median, range)	4.6 (0.1–18)	5.0 (0.4–17.2)	4.5 (1.1–7.9)
Male/female	179/136	42/42	3/2
Max. tolerable dose to mercaptopurine mg m^{-2} per day (mean ± s.d.) based on 310 patients of SR or HR	44.1 ± 15.3	30.7 ± 11.7	9.4 ± 5.7

Abbreviations: HR, high-risk; SR, standard-risk.

mercaptopurine dosage was reduced by 50%, then methotrexate would start to be reduced in dosage. The maximal dose in which the patient could tolerate for longer than 3 months till the end of continuation therapy was defined as 'maximal tolerable dose', which was expressed as mg m^{-2} per day. The genotyping for *TPMT* and *NUDT15* were also performed in normal bone marrow (BM) samples, obtained from 100 adults undergoing staging for localized lymphoma or evaluation for chronic immune thrombocytopenic purpura, which had morphologically normal BM. Written Informed consent was obtained from each adult patient examined. The study was approved by the Institutional Research Board of Chang Gung Memorial Hospital at Linkou (CGMF IRB no. 98-3768B and IRB no. 103-7559B).

Cell fraction

Mononuclear cells were isolated from ethylenediaminetetraacetic acid anticoagulated BM aspirates upon achieving complete remission using a Ficoll density gradient. The genomic DNA was extracted from mononuclear cells using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total genomic DNA was quantified using a NanoDrop ND-1000 spectrophotometer (Wilmington, DE, USA) at 260 nm.

TaqMan assays used for TPMT genotyping

Genotyping for *TPMT* (rs1142345) c.719 A>G (NM_0003673) was performed by means of TaqMan chemistry using the ABI Prism 7900 Sequence Detection system (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturers' instructions. The amount of DNA used in each individual assay was 10 ng. The TaqMan single-nucleotide polymorphism (SNP) genotyping assay part number was C_____19567_20. The PCR thermal cycling was as follows: initial denaturing at 95 °C for 10 min; 50 cycles of 92 °C for 15 s and 60 °C for 1 min 30 s.

Pyrosequencing for NUDT15 (rs116855232) c.415 C>T (NM_018283.1)

Pyrosequencing was performed as previously described.[18] Primers for PCR and subsequent primer for pyrosequencing were forward primer of TGGGTTCCTTGGGAAGAACTA, reverse primer of biotin-ATCCCACCAGA TGGTTCAGAT and sequencing primer of GCTTTTCTGGGGACT. The size of amplicon was 112 bp and sequences to be analyzed were GYGTTGTTT.

The target sequence was first amplified by PCR in which reverse primer was biotinylated for subsequent preparation of a single-strand DNA for pyrosequencing amplification, the biotinylated strand was captured on Streptavidin Sepharose beads and used as a template for pyrosequencing, which was prepared using the Vacuum Prep Workstation. Pyrosequencing was performed in a PyroMark ID (Biotage, Hilden, Germany) machine according to the manufacturer's instruction. Enzyme, substrate and nucleotides for pyrosequencing reactions were contained in PyroMark Gold Q96 Reagents kit (Qiagen). Allele frequency was quantified using the Qiagen software. Linearity of quantitative single-nucleotide variant detection by pyrosequencing was tested in triplicate by PCR products generated from various dilutions of 0% to 100% variants. The detection limit of our pyrosequencing assay was 2% ~3%.

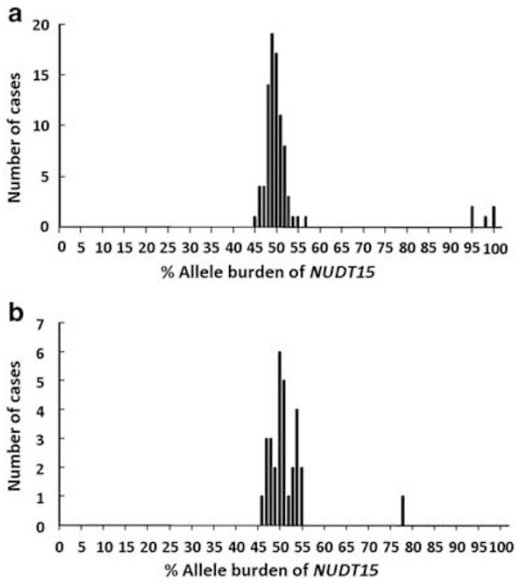

Figure 1. Allele burden of *NUDT15* (rs116855232) c.415 C>T. (**a**) Childhood ALL (TC $N=84$, middle cluster, TT $N=5$, right cluster) and (**b**) normal adult BM (TC $N=29$, middle cluster; TT $N=1$, right).

Statistical analysis

Correlations between categorical variables such as the maximal tolerable dose of mercaptopurine and SNPs were evaluated using the Kruskal–Wallis test. The doses of mercaptopurine and the SNP genotypes were compared using the χ^2- or Fisher's exact test. The duration of event-free survival (EFS) was defined as the time from the start of chemotherapy to any type of treatment failure (defined as relapse, death from any cause, development of second malignant neoplasm) or the day of last follow-up. Estimates of EFS were calculated according to the Kaplan–Meier method. Comparisons of estimated survival curves were analyzed by the log-rank test. In all analysis, the *P*-values were two-sided and considered statistically significant when values lower than 0.05. Statistical analysis was carried out by SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS

The laboratory and clinical results are summarized in Table 1.

Genotypes of NUDT15 rs116855232

In 404 ALL patients, 224 were boys and 180 girls. Five, 3 boys and 2 girls, were homozygous for *NUDT15* rs116855232 (TT), 84, 42 boys and 42 girls, were heterozygous (TC) (Figure 1a), and the remaining 315 were normal wild-type (CC) with an overall risk allele frequency of 11.6%. In 100 adult patients with normal bone marrow, one was homozygous, 29 were heterozygous (Figure 1b) and the remaining 70 were normal wild-type with an overall risk allele frequency of 15.5%. There was no statistical difference between pediatric ALL patients and adults with normal BM ($P=0.154$).

Genotyping of TPMT 719 A>G

Of the 404 ALL patients, none was homozygous, 13 were heterozygous (AG) and the remaining 391 were normal (AA) with an overall risk allele frequency of 1.6%. In the 100 normal adult BM samples, none was homozygous, one was heterozygous and the remaining 99 were wild-type with an overall risk frequency of 0.5%. No difference in the allele frequency was observed between children with ALL and adults with normal BM ($P=0.325$).

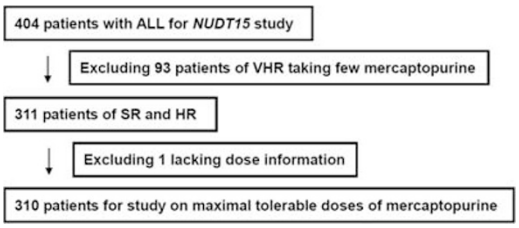

Figure 2. Consortium diagram of outcome analysis based on mercaptopurine treatment. HR, high-risk; SR, standard-risk; VHR, very high-risk.

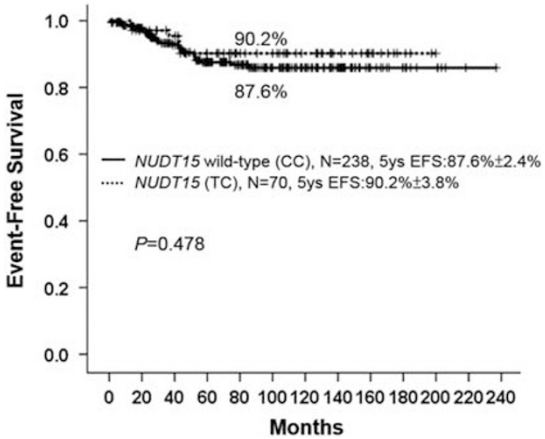

Figure 3. Event-free survival in pediatric acute lymphoblastic leukemia according to genotypes of *NUDT15*.

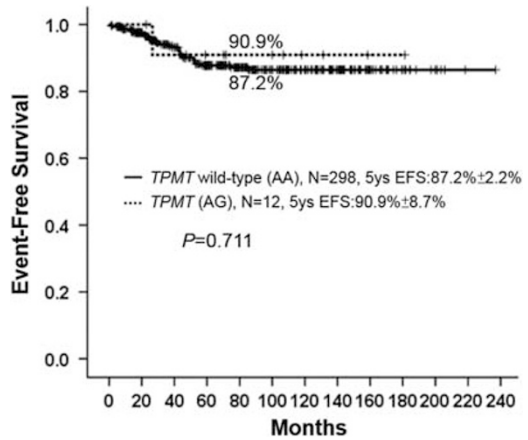

Figure 4. Event-free survival in pediatric acute lymphoblastic leukemia according to genotypes of *TPMT*.

Maximal tolerable dose of mercaptopurine according to genotypes of NUDT15 and TPMT

In the analysis of the maximal tolerable dose of mercaptopurine, 93 patients with very high-risk ALL were excluded because mercaptopurine and methotrexate constituted only one of four pairs of drugs given in rotation every 4 weeks, and hence mercaptopurine was scheduled to be given for only 7 days every 4 weeks.

In the other 311 patients with standard- or high-risk (SR or HR) ALL, one lacked data of mercaptopurine doses. The remaining 310 patients were subjected to outcome analysis based on

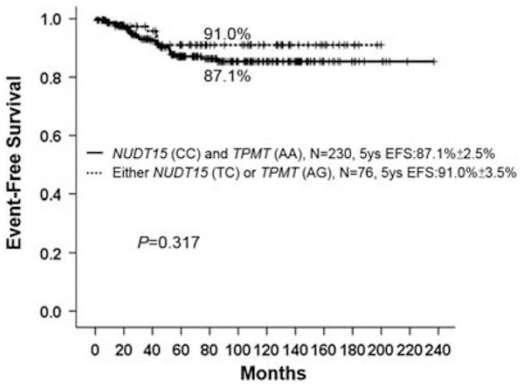

Figure 5. Event-free survival in pediatric acute lymphoblastic leukemia patients with *NUDT15* or *TPMT* heterozygous compared with wild-type.

Table 2. The treatment outcome of children with acute lymphoblastic leukemia according to *NUDT15* genotypes

Genotype	*Patient no.*	*Relapse*	*Toxic death*	*5-year EFS (%)*
CC	238	24 (10.1%)	2 (0.8%)	87.6 ± 2.4
TC	70	5 (7.1%)	1 (1.4%)	90.2 ± 3.8
TT	2	1	0	

Abbreviations: CC, wild type; EFS, event-free survival; TC, heterozygous; TT, homozygous.

mercaptopure (Figure 2). The maximal tolerable daily doses of mercaptopurine in *NUDT15* TT, TC and CC groups were 9.4 ± 3.7 (mean ± s.d.), 30.7 ± 11.7 and 44.1 ± 15.3 mg m^{-2}, respectively ($P < 0.0001$). The maximal tolerable dose of mercaptopurine in *TPMT* AG and AA were 31.4 ± 10.2 and 41.2 ± 15.8 mg m^{-2}, respectively ($P = 0.034$). The maximal tolerable daily dose of mercaptopurine in double heterozygous for both *NUDT15* and *TPMT* ($n = 4$) was 32.7 ± 14.3 mg m^{-2} per day compared with single heterozygous for *NUDT15* or *TPMT* ($n = 76$) of 30.0 ± 11.7 mg m^{-2} per day ($P = 0.655$), the latter was significantly different from the dose 44.6 ± 15.3 mg m^{-2} per day in wild-type for both *NUDT15* and *TPMT* ($n = 231$) ($P < 0.0001$).

Treatment outcome according to genotypes of NUDT15 and TPMT

The ratio of SR to HR in *NUDT15* TC was 1.8 (45/25) and in CC was 1.43 (140/98), respectively. The 5-year EFS, which was based on 310 patients of SR or HR groups, excluding the one lacking information (Figure 2), did not differ significantly between patients with heterozygous and wild-type *NUDT15* ($P = 0.478$, Figure 3). Of the two patients with *NUDT15* TT treated in SR arm, one relapsed and one remained in remission.

No difference in EFS was observed between patients with heterozygous and wild-type *TPMT* ($P = 0.711$, Figure 4). There was also no significant difference in 5-year EFS between patients heterozygous for one of the *NUDT15* or *TPMT* gene and wild-type ($P = 0.317$, Figure 5). The four patients carrying double heterozygous of both *NUDT15* and *TPMT* seemed not have an inferior EFS compared with heterozygous for single gene, one of them relapsed with no toxic death. The treatment outcome including relapse, toxic death and EFS according to *NUPT15* genotypes is shown in Table 2.

DISCUSSION

Mercaptopurine and 6-thioguanine share a similar metabolic pathway. Mercaptopurine is anabolized by hypoxanthine phosphoribosyltransferase to thioinosine monophosphate and finally to mono-, di- and triphosphates of 6-thioguanosine, named 6-thioguanine nucleotides, which interfere DNA and RNA synthesis and are important for cytotoxic effects of mercaptopurine and 6-thioguanine.[19] Mercaptopurine is catabolized by xanthine oxidase to thiouric acid and by methylation from TPMT to methylmercaptopurine, which can inhibit *de novo* purine synthesis.[9] *NUDT* encodes a nucleotide diphosphatase,[20] and degrades oxidized purine nucleoside triphosphatases by dephosphorylation to prevent its incorporation to DNA.[21] NUDT15 is a safeguard to remove the oxidatively damaged guanine nucleotides from cells to minimize DNA damage and avoid further apoptosis.[21] Yang *et al.*[15] showed that the viability of Jurkat cells transfected by mutant *NUDT15* construct was lower than that of control cells transfected by wild-type construct probably owing to an increased apoptosis.

The frequency of risk allele *NUDT15* rs116855232 was 11.6% in ALL patients and 15.5% in adults with normal BM in this study of Taiwanese. Yang *et al.*[15] performed an immunochip-based two-stage association study in 978 Korean patients with Crohn's disease treated with thiopurines and identified a nonsynonymous SNP in rs116855232, which was closely related to thiopurine-induced leukopenia. They reported that the frequency of risk allele in Koreans was 10.4%, in Japanese 7% and in Chinese 13%, compared with 2% in an admixed American population, and very low in the individuals of European descent.[15] Yang JJ *et al.*[22] found that in children with ALL, the risk alleles of *NUDT15* were 9.8% in East Asians, 3.9% in Hispanics, 0.2% only in Europeans and monomorphic in Africans. We examined a Taiwanese population and also demonstrated a reduced tolerance to mercaptopurine among pediatric ALL patients with homozygous or heterozygous *NUDT15* risk allele, similar to the findings of Yang SK *et al.*[15] and Yang JJ *et al.*[22] In contrast to *NUDT15*, the overall risk allele frequency of *TPMT* polymorphism was low in our Taiwanese population, 1.6% in ALL patients and 0.5% in 100 adults with normal BM. Our previous study in 249 Taiwanese revealed an allelic frequency of 0.6%.[14] Hence, *TPMT* SNP has little contribution to the poor tolerance of Taiwanese population to mercaptopurine. Obviously, the high frequency of risk allele in *NUDT15* rs116855232 in Taiwanese can largely explain the intolerance to mercaptopurine in Taiwanese children with ALL. Actually, Yang JJ *et al.* [22] also demonstrated that all children with homozygote of either *NUDT15* or *TPMT* variant, or heterozygote of both needed at least 50% reduction in mercaptopurine dose.

Preemptive test to define the genotypes of *NUDT15* is strongly recommended for Taiwanese children and probably all the Far East children with ALL. The dosage of mercaptopurine can be prospectively reduced in patients with *NUDT15* polymorphism to prevent excessive hematopoietic toxicity and severe infections while improving treatment outcome.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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