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ORIGINAL RESEARCH ARTICLE

Effect of *GSTP1* polymorphism on efficacy and safety of cyclophosphamide aggressive therapy in lupus nephropathy patients

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Abstract

Background Lupus nephritis (LN) occurs in up to 60% of adults with systemic lupus erythematosus (SLE) and is a predictor of poor survival. Cyclophosphamide (CYC) is regarded as the most effective immunosuppressive medication to improve survival for patients with LN.

Objective This prospective hospital-based study was conducted to identify the effect of glutathione S transferase Pi-1 (*GSTP1*) genotypes on the efficacy and safety of CYC aggressive therapy.

Methods We enrolled SLE nephropathy patients admitted to the Department of Rheumatology of the 500-bed Yangon Specialty Hospital (YSH), Yangon, Myanmar, who received CYC aggressive therapy for 6 months according to treatment guidelines for SLE patients with renal involvement. The frequencies of I/I, I/V and V/V *GSTP1* genotypes were determined using the polymerase chain reaction-restriction fragment length polymorphism method. The efficacy of CYC aggressive therapy between LN patients with wild *GSTP1* (I/I) and those with polymorphic *GSTP1* (I/V or V/V) genotypes was evaluated by comparing 24-h urinary protein levels and assessing the remission rates at 3 and 6 months after initiation of CYC. CYC-related myelotoxicity was assessed by reviewing complete blood picture results on the 10th day after CYC treatment.

Results In total, 95 eligible patients were recruited. The frequencies of I/I, I/V and V/V *GSTP1* genotypes were 54.7, 41.1 and 4.2%, respectively. At 3 and 6 months after CYC treatment, mean 24-h urinary protein had significantly decreased from baseline in both wild and polymorphic genotype groups ($p < 0.001$). No significant differences were seen between the wild and polymorphic genotype groups with regard to changes in 24-h urinary protein levels, remission at 3 and 6 months or myelotoxicity.

Conclusion CYC aggressive therapy had similar efficacy and caused no significant differences in myelotoxicity in wild *GSTP1* (I/I) and polymorphic *GSTP1* (I/V or V/V) genotypes in patients treated according to YSH guidelines for SLE patients with renal involvement.

Introduction

Lupus nephritis (LN) occurs in up to 60% of adults with systemic lupus erythematosus (SLE) and predicts poor survival [1]. In 2016, a total of 796 patients with SLE were admitted to the Department of Rheumatology of the 500-bed Yangon Specialty Hospital (YSH) in Myanmar, accounting for 60% of all admissions to this department.

LN is the most frequent severe visceral condition affecting patients with SLE [2]. Renal involvement in Asian SLE patients is 21–65% at diagnosis and 40–82% at follow-up [3]. The presence of nephropathy significantly reduces survival to ≈ 88% at 10 years, with lower survival rates in African Americans. Cyclophosphamide (CYC) is

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viewed as the most effective of the immunosuppressive medications for lupus glomerulonephritis [4].

Many factors may play an important role in the response to CYC treatment. Among them, genetic factors may have considerable influence on the response to CYC immunosuppressive regimens. The initial activation of CYC is 4-hydroxylation to form 4-hydroxy-cyclophosphamide (4-OH-CYC) and then to active phosphoramide mustard and the byproduct toxic acrolein [5]. These toxic metabolites of CYC also gain entry to normal tissues, including the gastrointestinal tract and bone marrow, where they induce host organ injuries in many patients [6].

Detoxification of 4-OH-CYC, phosphoramide mustard and acrolein may occur via intracellular conjugation with glutathione mediated by the enzyme glutathione S-transferase (GST) in hepatocytes. A considerable number of genetic polymorphisms among the soluble GSTs have been described in humans. Variations in *GST* alleles are very common and contribute substantially to inter-individual differences in drug metabolism [7]. Polymorphisms in any of the GST genes produce significant alterations in the metabolism of chemotherapeutic agents and carcinogens. The GSTs involved in CYC detoxification include GSTM1, GSTT1, GSTA1 and GSTP1. Reduction in enzymatic activity due to polymorphism of these enzymes leads to decreased detoxification of active CYC and prolonged exposure to the drug. This can increase the risk of adverse drug effects but, in theory, may also lead to improved survival in cancer patients [8].

A single nucleotide polymorphism (SNP), the single-nucleotide substitution adenine 313 guanine, results in an amino acid change at codon 105 (isoleucine [Ile] to valine [Val]) at *GSTP1* that substantially diminishes the enzyme activity of the GSTP1 protein [6]. A French study assessed the hypothesis that genetic polymorphisms of GSTs could impact remission and adverse drug reactions related to CYC in LN patients; their multivariate analysis indicated that the Ile to 105Val *GSTP1* genotype was an independent factor for poor renal outcome [9].

A study assessing therapeutic success in patients with acute lymphoblastic leukemia with polymorphic GST genes showed that a threefold decrease in risk of relapse was associated with the Val/Val genotype compared with the combined category (Ile/Val and Ile/Ile) [10]. Similarly, women who were homozygous for the variant *GSTP1* Val105 allele had a 60% reduction in mortality risk and improved survival after chemotherapy compared with women who were homozygous for the Ile allele [11]. Improved overall survival after cancer chemotherapy has also been shown with *GSTP1* Val/Val or Ile/Val in comparison with *GSTP1* Ile/Ile genotypes in patients with multiple myeloma and Hodgkin's lymphoma [12, 13].

Pharmacogenetics enables the prediction of better therapeutic response while helping to avoid toxicity according to the patient's genotype and has the potential to lead to personalized medicine in the future. Although the effect of *GSTP1* polymorphism on response to CYC, including chemotherapy among cancer patients, has been previously researched, very little research has been conducted on this gene polymorphism in SLE patients. The interplay between the dose–genetic factor relationships of CYC in these patients has not been fully elucidated. Limited evidence-based data make it difficult to optimize the dose and treatment regimens when treating SLE patients with CYC. This study aimed to identify the frequencies and the effects of the *GSTP1* polymorphism on response to CYC aggressive therapy among SLE nephropathy patients in Myanmar according to YSH guidelines for SLE with renal involvement [14]. Our findings provide insight into the variability of drug responses among SLE nephropathy patients receiving CYC aggressive therapy and will be beneficial for clinicians when modifying CYC therapy in these patients.

Materials and methods

This hospital-based prospective comparative study was conducted in SLE nephropathy patients who were admitted to the Department of Rheumatology, YSH, from January 2016 to February 2017. The study was approved by the Research and Ethics Committee of University of Medicine 1, Yangon. We included patients of any age and both sexes who were treated according to guidelines for CYC aggressive therapy for renal involvement and who gave informed consent. Patients with serum creatinine > 300 µmol/L and relapse cases were excluded from the study because dosage modification and response in these patients may differ from that in patients naïve to CYC therapy. See Fig. 1 for the flowchart of the study procedure.

SLE patients with active renal involvement are defined clinically as those with persistent proteinuria > 0.5 g/day and/or cellular casts including red blood cells (RBCs), hemoglobin, granular, tubular, or mixed [4]. These patients received CYC aggressive therapy because they had a high disease activity (SLE Disease Index [SLEDI]) score and the presence of extrarenal manifestations apart from renal involvement. The dosage of intravenous CYC in the YSH guideline for induction therapy is 600 mg for 1 day (10 mg/kg/month; not more than 600 mg). The corticosteroid was intravenous methyl prednisolone (MP) 500 mg for 3 days and oral prednisolone 1 mg/kg/day daily for 4 weeks. The dose of intravenous CYC 10 mg/kg is ≈ 0.4 g/m^2. At the consolidation phase of 5 months, the monthly intravenous CYC dose is also 10 mg/kg (not more than 400 mg/month) and monthly intravenous MP was 250 mg [14].

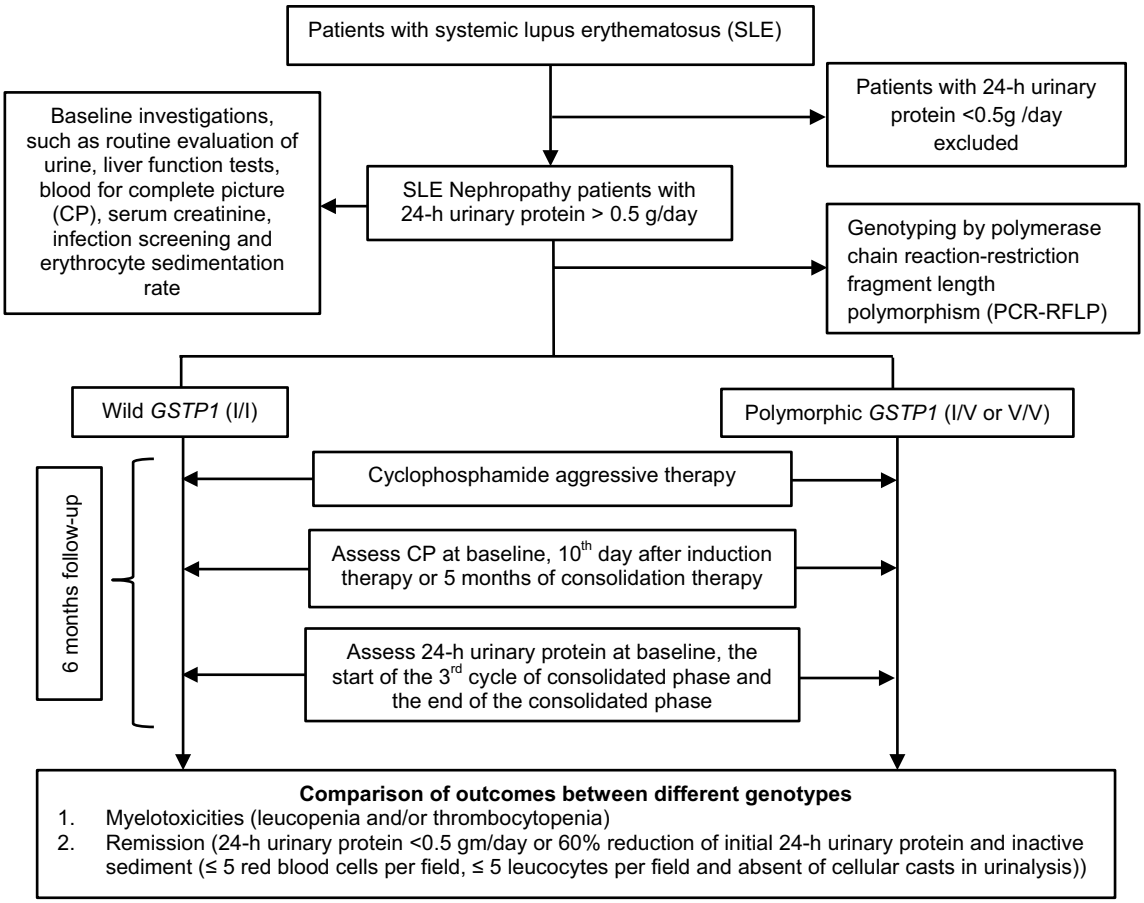

Fig. 1 Flowchart of the study procedure

Complete remission from lupus nephropathy was determined with 24-h urinary protein < 0.5 g/day or 60% reduction and inactive sediment (≤ 5 RBC per field, ≤ 5 leukocytes per field and absence of cellular casts in urinalysis) [14]. Urine for 24-h urinary protein analysis was collected at the time of diagnosis as a baseline parameter; response to therapy was then monitored by measuring the 24-h urinary protein again at the start of the 3rd cycle (at the end of the 3rd month of therapy) and the last cycle of the consolidation phase (7th month of therapy).

A common adverse effect of CYC therapy is myelosuppression. This was monitored by reviewing the results of blood for complete picture (CP) results for myelosuppression. Myelotoxicity, or bone marrow suppression or myelosuppression, can be regarded as the decrease in production of leukocytes and/or thrombocytes (leukopenia and/or thrombocytopenia). Leukopenia is defined as white blood cell (WBC) count $< 4.0 \times 10^3/\mu L$. Thrombocytopenia is defined as a platelet count $< 150 \times 10^3/\mu L$ (according to National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0) [15]. CP results were recorded at baseline, on the 10th day after the start of the induction phase, or after the monthly consolidation phase for 5 months because leukocyte counts reached a nadir between the 9th and 12th days. We reviewed these results to compare the clinical outcome parameters (proteinuria/complete remission) and risk of myelosuppression between *GSTP1* (I/I) (wild type) and *GSTP1* (I/V or V/V) (polymorphic) genotypes.

Genotyping of *GSTP1* polymorphism

For genetic polymorphism, 2 mL of whole blood was collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes. Polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) was conducted to detect *GSTP1* genotypes. Genomic DNA was isolated from peripheral blood leukocytes using a commercially available kit (QIAGEN) in accordance with the manufacturer's instructions. The extracted DNA was stored at − 20 °C until analysed. The PCR reaction mixture (25 µL) contained 2 µL (10 µM) each of forward primer (5′-GAG GAAACTGAG ACCCACTGAG-3′) and reverse primer (5′-AGCCCCTTTCTTTGTTCAGCC-3′), 12.5 µL of Taq PCR Master Mix (QIAGEN, USA), 2 µL (30 ng) of

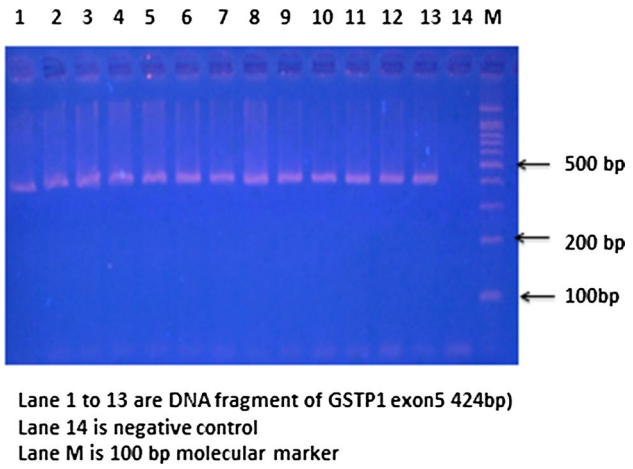

Fig. 2 Electrophoresis pattern of polymerase chain reaction for detection of *GSTP1* gene on agarose gel

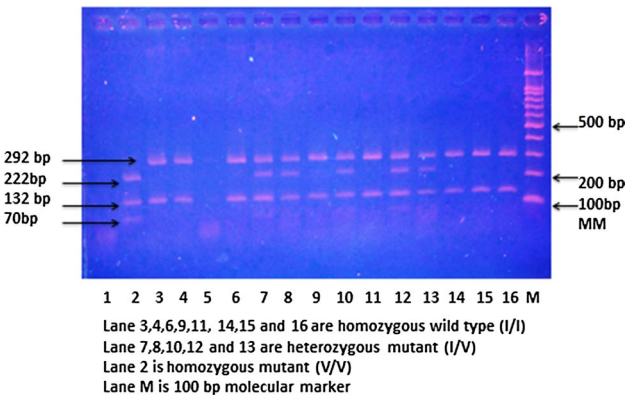

Fig. 3 Electrophoresis pattern of restriction fragment length polymorphism for detection of *GSTP1* polymorphism on agarose gel

template DNA and 6.5 µL of RNase free water. Amplification was performed with initial denaturation at 94 °C for 5 min followed by 35 cycles at 94 °C for 30 s, 64 °C for 30 s, and 72 °C for 30 s [16]. After a final extension at 72 °C for 5 min, PCR products were separated on 2% agarose gel and stained with ethidium bromide to detect a 424 bp DNA fragment for *GSTP1* exon 5 (Fig. 2).

RFLP was conducted as follows: *GSTP1* DNA fragments were digested for 3 h at 55 °C using the restriction enzyme BsmaI (New England Biolabs, England) 0.5 µL (5U), 10 × Cut Smart buffer 2.5 µL, RNase free water 12 µL, and PCR product 10 µL. *GSTP1* DNA fragments were classed as wild type (I/I) by two bands (292 and 132 bp), heterozygous genotype (I/V) by four bands (292, 222, 132 and 70 bp) and homozygous genotype (V/V) by three bands (222, 132 and 70 bp) on an agarose gel (Fig. 3).

Statistical analysis

Frequency distribution was used to describe the socio-demographic characteristics and polymorphism of SLE patients. As 24-h urinary protein data were not normally distributed, they were summarized as median (interquartile range [IQR]), and results were compared with non-parametric tests (e.g., Wilcoxon signed rank test and Mann–Whitney *U* test). Remission rate and myelotoxicities between two genotypes were compared using the Chi squared test. Statistical Package for Social Science (SPSS) software version 16.0 was used for data entry and statistical analysis. A p value < 0.05 was considered statistically significant.

Results

In total, 95 SLE nephropathy patients who received CYC aggressive therapy were recruited in this study. During the study period, 14 patients withdrew from the study (at the 1-month, 3-month and 6-month follow-up periods); however, *GSTP1* polymorphism was identified among all 95 patients. Almost all (92 [96.8%]) patients were female, and patient age ranged from 21 to 40 years.

The frequencies of I/I, I/V and V/V *GSTP1* genotypes were 54.7, 41.1 and 4.2%, respectively. The electrophoresis pattern for genotyping can be seen in Figs. 1 and 2. Table 1 shows the changes in 24-h urinary protein levels in wild *GSTP1* (I/I) and polymorphic *GSTP1* (I/V or V/V) genotypes in LN patients from baseline to 3 months and 6 months after CYC aggressive therapy. The results showed significant decreases in 24-h urinary protein in both groups after 3 and 6 months of CYC aggressive therapy (all $p < 0.001$ vs. baseline).

No statistically significant differences were found in 24-h urinary protein levels between wild *GSTP1* (I/I) and polymorphic *GSTP1* (I/V or V/V) genotypes in LN patients at baseline or at 3 and 6 months after CYC aggressive therapy (Table 1).

The efficacy of CYC aggressive therapy was also assessed by remission. Remission did not differ significantly between wild *GSTP1* (I/I) and polymorphic *GSTP1* (I/V or V/V) genotypes at the 3rd and 6th month after CYC therapy (Table 2).

The incidence of myelotoxicity (total WBC counts and/or platelet counts) between wild *GSTP1* (I/I) and polymorphic *GSTP1* (I/V or V/V) genotypes at the 10th day after CYC treatment was compared by reviewing blood for CP results. No significant differences in myelotoxicity between these groups were seen (Table 3).

Table 1 Changes in 24-h urinary protein in wild *GSTP1* (I/I) and polymorphic *GSTP1* (I/V or V/V) genotypes at 3 and 6 mo after cyclophosphamide aggressive therapy

Genotype	Median 24-h urinary protein in mg/day [IQR] (no. of missing values)		
	Baseline	3 mo after CYC aggressive therapy	6 mo after CYC aggressive therapy
Wild *GSTP1* (I/I) [$n=49$]	1961 [4800–1031.5]	583* [1603–206]	373.5* [1204.5–133.3] (5)
Polymorphic *GSTP1* (I/V or V/V) [$n=43$]	2648 [5157–1079]	614* [1912–225.5] (3)	434* [1424.5–146] (6)

CYC cyclophosphamide, *GSTP1* glutathione S-transferase Pi-1, *IQR* interquartile range, *mo* months

*$p<0.001$ vs. baseline (Wilcoxon signed rank test)

Median 24-h urinary protein levels in wild *GSTP1* vs. polymorphic *GSTP1* genotypes: no significant between-group differences; $p=0.625$ at baseline; $p=0.879$ at 3 mo; $p=0.787$ at 6 mo (Mann–Whitney *U* test)

Table 2 Remission rates in wild *GSTP1* (I/I) and polymorphic *GSTP1* (I/V or V/V) genotypes at 3 and 6 mo after CYC therapy

Time after CYC aggressive therapy	No. of patients with remission (%)		
	Wild *GSTP1* (I/I)	Polymorphic G*STP1* (I/V or V/V)	*p*-value[a]
3 mo	23/44 (52.3)	18/33 (58.1)	0.620
6 mo	25/36 (69.4)	17/27 (63)	0.589

[a]Chi squared test

CYC cyclophosphamide, *GSTP1* glutathione S-transferase Pi-1, *mo* months

Table 3 Leukopenia, thrombocytopenia and myelotoxicity rates in wild GSTP1 (I/I) and polymorphic GSTP1 (I/V or V/V) genotypes at 10 days after CYC therapy

Condition	No. of patients (%)		
	Wild *GSTP1* (I/I)	Polymorphic G*STP1* (I/V or V/V)	*p*-value[a]
Leukopenia	1/34 (2.9)	2/33 (6.1)	0.61
Thrombocytopenia	2/34 (5.9)	1/33 (3.0)	1.000
Myelotoxicity	3/34 (8.8)	3/33 (9.1)	1.000

[a]Fisher's exact test

CYC cyclophosphamide, *GSTP1* glutathione S-transferase Pi-1

Discussion

Sharma et al. [17] found I/I, I/V and V/V frequencies of 60.79, 34.25 and 4.96% in an Asian population; we found similar distributions in the present study. Regarding the efficacy of CYC, both groups showed significantly decreased 24-h urinary protein, but patients with wild *GSTP1* (I/I) genotype group had a better response to CYC therapy in the early months of therapy.

Sigdel et al. [18] conducted a similar study in SLE nephropathy patients in Nepal. In their study, the dose of intravenous CYC was 500 mg monthly for 6 months, and intravenous MP (0.5–1 g) was used for only 3 days. The remission criteria were set at 24-h urinary protein < 200 mg/day. Of the 34 patients, 18 (43.9%) achieved complete remission at 3 and 6 months after treatment [18]. Even though the remission criteria differed slightly from those in the present study, the remission rate was comparable.

A retrospective study by Valim et al. [19] reviewed the treatment response of 35 LN patients in Brazil who underwent induction therapy with high-dose CYC 0.5–1 g/m^2 monthly for 6 months. The results showed that 26 (74%) patients achieved remission right after the induction period [19]. This study showed better clinical responses, which may be because their dosage range was higher than in the present study; higher dosages may provide higher response rates. It can be concluded that the efficacy of CYC therapy in LN patients in the present study was as good as that of these other studies. Kumaraswami et al. [20] conducted a study of epistatic interactions among *CYP2C19*2*, *CYP3A4* and *GSTP1* on CYC therapy in LN patients (induction therapy was intravenous pulse CYC 750 mg/m^2 monthly for 6 months) and reported that *CYP2C19*2*, *GSTP1* and *CYP3A5*3* have synergistic influences on CYC failure.

However, in the present study, the results of mean 24-h urinary protein and remission at 3 and 6 months after CYC therapy did not differ significantly between wild *GSTP1* (I/I) and polymorphic *GSTP1* (I/V or V/V). No effect of *GSTP1* polymorphism on CYC response was seen.

Some studies have investigated the effects of *GSTP1* polymorphism in association with toxicities from CYC-containing cancer chemotherapy. In 2017, Ma et al. [21] conducted a meta-analysis to evaluate the influence of *GSTP1* polymorphism on toxicity outcomes in breast cancer patients and

reported that the *GSTP1* polymorphism was associated with increased toxicities, especially in patients treated with chemotherapy ± surgery. Conversely, another study [22] reported that *GSTP1* variant 105 I/V was related to a reduced risk of neutropenia and leukopenia in breast cancer patients receiving cancer chemotherapy.

With regards to the treatment of SLE with CYC immunosuppressive therapy, reports regarding the risk of toxicities in *GSTP1* polymorphic patients are limited, although infection and leukopenia due to myelotoxicities have always been major limiting factor in lupus therapy [18]. When used as cancer chemotherapy, the initial course of CYC for patients with no hematologic deficiency usually consists of 40–50 mg/kg given intravenously in divided doses over a period of 2–5 days. Other intravenous regimens include 10–15 mg/kg every 7–10 days or 3–5 mg/kg twice weekly [23]. In many studies, the myelotoxic effect of *GSTP1* polymorphism was determined in combination chemotherapy. The general rule for combination chemotherapy is that drugs are selected on the basis of not having overlapping toxicity; however, nearly all chemotherapy agents suppress bone marrow [24]. Therefore, the occurrence of myelotoxicities may be additive in these studies.

In the treatment of SLE, lower dosages of CYC are generally used, at a frequency of every 2 weeks or monthly. In the present study, the CYC dosage regimen used in both induction and maintenance therapy was only 10 mg/kg monthly. As dosages may differ, so too may the degree of myelotoxicity between these treatment regimens. The CYC dose of 10 mg/kg (0.4 g/m^2) was lower than the standard dose used in the American College of Rheumatology treatment guideline; therefore, the risk of myelotoxicity could not be adequately demonstrated.

Zhong et al. [16] conducted a study regarding the toxic effects of *GSTP1* polymorphism in newly diagnosed SLE patients. Patients were administered induction therapy of intravenous CYC 0.5–0.75 g/m^2. Patients were then closely monitored for toxicity for 2 weeks after therapy initiation. The authors found that the incidence of myelotoxicity was 5.7-fold higher in patients with *GSTP1* (I/V or V/V) genotypes than in those with the *GSTP1* wild-type genotype (I/I). In their study, the occurrence of myelotoxicity was significantly higher in *GSTP1* polymorphic patients receiving higher CYC doses (> 1.0 g) than in those in the wild-type genotype group [16]. The dose of CYC used in the present study fell into the lower dosage group used in the study by Zhong et al. [16]. In their study, the therapy given to SLE patients was intravenous CYC only, and corticosteroid therapy was spared [16]. In the present study, corticosteroids were used during the entire 6-month treatment period, and much literature concerning corticosteroid-induced leukocytosis has been published. As a result, the risk of myelotoxicity could not possibly be demonstrated.

A limitation of this study is that the patients were unable to participate in the later months of follow-up to allow assessment of efficacy. The development of myelosuppression during treatment is also influenced both by drug therapy and by patient characteristics, including age, general condition and comorbidities, which we were unable to analyze in this study. Therefore, the effects of *GSTP1* polymorphism in the long-term remission of LN with CYC aggressive therapy should be evaluated, and the effects of *GSTP1* polymorphism on myelotoxicities in LN patients receiving higher dosages of CYC than were used in this study.

Conclusion

We conclude that CYC aggressive therapy when used according to the YSH guidelines has similar efficacy and caused no significantly different myelotoxicities between wild-type *GSTP1* (I/I) and polymorphic *GSTP1* (I/V or V/V) genotypes. Remission from nephropathy using guideline-based CYC therapy had acceptable efficacy and toxicities.

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Compliance with ethical standards

Ethical approval This study was approved by the Research and Ethics Committee of University of Medicine 1, Yangon, and was registered at Thai Clinical Trial registry with the trial registration number TCTR20180828012. All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individuals participating in the study.

Conflicts of interest K Khine Thu, Aye Aye Lwin, Khin Than Maw, Lei Lei Htay, Khin Mar Myint, Myat Myat Soe, Ye Htut Linn, Chit Soe, and Nang Hla Hla Win have no conflicts of interest that are directly relevant to the content of this article.

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