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Polymorphisms of GSTT1 and GSTM1 Genes in Diffuse Large B Cell Lymphoma Egyptian Patients

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ABSTRACT

Background: Evidence, although not so extensive, did show that genetic variations in glutathione S-transferase (GST) might be associated with risk of developing lymphoma; overall and/or subtype. Genetic data on the contribution of GST genes in the prognosis of lymphomas is scarce even in occupationally exposed populations.

Aim: The study aimed to investigate the influence of polymorphisms in GSTM1 and GSTT1 genes on both the risk and prognosis of diffuse large B-cell lymphoma (DLBCL).

Subjects and Methods: The study included newly diagnosed 83 DLBCL cases and 89 age and gender matched control subjects. DLBCL patients underwent IPI scoring, CHOP treatment and follow-up for treatment outcome, relapse/progression and death. For all subjects, GSTM1 and GSTT1 genotyping was performed by a multiplex PCR protocol.

Results: We found 2.35 fold increase in the risk of DLBCL associated with GSTT1-null genotype (OR=2.35, 95% CI: 1.02-5.40, p=0.04). Three times increased risk in individuals with the GSTM1/T1-double null genotype (OR3.06, 95% CI: 1.04-8.95, p=0.03) compared with both GSTM1 and GSTT1 genes undeleted (wild genotype) was observed. Patients; overall and those with favorable IPI (<3), showing one GST-null genotype and those showing GSTM1/T1-double null genotype significantly had better progression free survival (PFS) and overall survival (OS) when compared with those showing both GST wild genes. Multivariate analysis showed that the presence of at least one GST-null genotype was associated with a 60% reduced risk of relapse/progression and 71% reduced risk of death.

Conclusion: Our results have shown a role for the GSTT1-null genotype and the GSTM1/T1-double null genotype as risk factors for DLBCL. The presence of at least one GST-null genotype tended to have a positive prognostic value for DLBCL patients independent from both the IPI score and the treatment outcome. Large studies on rituximab plus CHOP treated patients will be needed to either support or modify these findings.

Key Words: *GSTT1 – GSTM1 – DLBCL – Egyptian.*

INTRODUCTION

B-cell lymphomas comprise the majority of cases of non-Hodgkin lymphomas (NHL) and, of these, diffuse large B-cell lymphoma (DLBCL) is the major subtype. DLBCL is the most common of the aggressive NHL **[1]**.

For the vast majority of patients, the etiology of diffuse large B-cell lymphoma is unknown. Factors thought to potentially confer increased risk include immunosuppression (including AIDS, and iatrogenic etiologies in the setting of transplantation or autoimmune diseases), ultraviolet radiation, pesticides, hair dyes, and diet **[2]**. Implicated pesticides include phenoxyacetic acid herbicides, organophosphate insecticides, triazine herbicides and fungicides **[3]**.

Established adverse prognostic factors for Non-Hodgkin lymphoma (NHL), as delineated in the International Prognostic Factor Index (IPI), include older age at diagnosis, higher tumor stage, poor performance score, extranodal involvement, and above-normal lactate dehydrogenase **[4]**.

Glutathione S-transferases (GSTM1, GSTT1, GSTP1) are involved in the detoxification of a wide range of carcinogens, including benzene, organochlorine compounds, organophosphate pesticides, tobacco smoke, chemotherapeutic agents, and reactive oxygen species **[5]**.

Polymorphisms in genes that code various types of GSTs manifest as decreased or lack of enzyme activity **[6]**, prompting the hypothesis that allelic variants may be associated with an

impaired detoxification capacity as well as drug metabolism. Subsequently, they may play a role in increased susceptibility to cancer and may also influence tumor-response to anticancer drugs.

Evidence, although not so extensive, did show that genetic variations in GST might be associated with risk of developing NHL overall and/or subtype **[7,8,9]**. Genetic data on the contribution of GST genes in the prognosis of lymphomas is scarce even in occupationally exposed populations.

Genetic polymorphism that confers susceptibility to or protection from certain cancer type may be quite different in different ethnic populations and the existence of racial and ethnic disparities in healthcare access and outcomes is well-documented.

To ascertain whether there is any association between GST gene polymorphisms and DLBCL risk in Egyptian population, we determined the frequencies of GSTT1 and GSTM1 gene polymorphisms in Egyptian population inhabiting a farming locality (Menofiya governorate) and demonstrating a known insecticide exposure and compared them with the frequencies found in age and gender matched control subjects inhabiting the same locality. Moreover, we studied the implications of these gene polymorphisms on DLBCL patients' prognosis and survival.

SUBJECTS AND METHODS

Subjects:

The study included 83 DLBCL patients (median age 60 years, range 18-84 years; 26 females and 57 males). Forty five bone marrow samples (withdrawn during routine staging of patients) and 38 Peripheral blood samples were obtained at the time of initial diagnosis. All were treated with standard chemotherapy regimen (CHOP) Doxorubicin 50mg/m^2 on day 1, Cyclophosphamide 750mg/m^2 on day 1, Vincristine 1.5mg/m^2 on day 1, Prednisone 100mg on day 1-5. Cycle was repeated every 3 weeks. Patients who achieved complete remission by the 4th cycle continued up to six cycles (for stage I and II disease) or eight cycles (for advanced disease). Patients were evaluated every 2 cycles and after completion of all planed cycles of CHOP to score response according to international working group criteria **[10]**. Patients who did not enter into complete remission or those who relapsed were shifted to second line chemotherapy; DHAP (dexamethasone, cytarabine and cisplatin) or ICE (ifosfamide, carboplatin and etoposide). None of the patients underwent bone marrow transplantation after relapse. Peripheral blood samples from 89 age and gender matched control subjects (median age 57 years, range 18-85 years; 26 females and 64 males), were also obtained. Informed consent was obtained from all subjects included in the study. The procedures followed were in accordance with the ethical standards of the responsible institutional committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 1983.

GSTT1 and GSTM1 genotyping:

DNA was extracted from bone marrow / peripheral blood leukocytes using DNA purification kit (QIAamp®DNA Blood Mini Kit, Qiagen, 28159 Avenue Stanford, Valencia, USA). DNA was quantified spectrophotometrically at 260nm and 280nm and stored at –20°C. The DNA quality was checked using *ß*-globin gene amplification (indicated by a 273-bp fragment) as a control gene using sense 5'-CAA CTT CAT CCA CGT TCA CC 3' and antisense 5'-GAA GAG CCA AGG ACA GGT AC 3'. Genotyping of the GSTM1 and GSTT1 loci was carried out by a multiplex PCR reaction in a Perkin Elmer thermal cycler (Gene amp, PCR system 2400 PERKIN ELMER, version 2.11, USA). The genotypes were analyzed according to the protocol of Arand et al. **[11]**. Genomic DNA was amplified by using 2 sets of primers: GSTM1 (F): 5'-GAA CTC CCT GAA AAG CTA AAG C-3'; GSTM1 (R): 5'GTT GGG CTC AAA TAT ACG GTG G-3'; GSTT1 (F): 5'-TTC CTT ACT GGT CCT CAG ATC TC-3'; GSTT1(R): 5'-TCA CCG GAT CAT GGC CAG CA-3'. Reaction mixture was 50µL containing 1µg DNA, 200µM dNTPs, 2.5U Taq polymerase (DreamTaq™ DNA Polymerase, Fermentas Inc. USA, 798 Cromwell Park Drive, Glen Burnie, MD 21061), 0.5µM of GSTM1 and 0.3µM of GSTT1 primers. A total of 40 PCR cycles with denaturation at 94°C for 1 minute, annealing at 59°C for 1 minute, and extension at 72°C for 1 minute were conducted. An initial DNA denaturation at 95°C and a final extension at 72°C were carried out for 5 minutes each. The PCR product was then subjected to

electrophoresis on a 2% agarose gel. The presence of bands of 480 and 215bp was indicative of the GSTT1 and GSTM1 genotypes respectively, whereas the absence indicated the null genotype for that gene (Fig. 1). This method cannot differentiate between the wild type and heterozygous state. It determines only homozygous deletion of GSTM1 and/or GSTT1 genes.

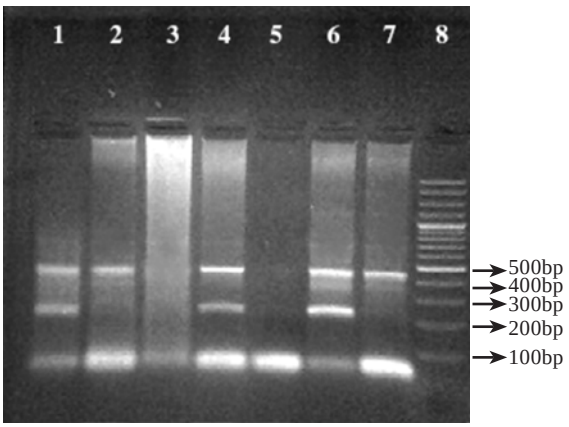

Fig. (1): Agarose gel electrophoresis of PCR products after amplification. Lane 8 shows the DNA marker, lane 5 shows non-template control, lanes 1,4 and 6 show both GSTM1 and GSTT1 undeleted genotype, lanes 2 and 7 show GSTM1-null genotype, lane 3 shows GST double-null genotype.

Statistical analysis: The frequency of the various enzyme genotypes in NHL cases and controls were compared using Fisher's exact tests. Odds ratios (OR) express the relative risk of NHL with a specific genotype. Survival curves were estimated using the Kaplan-Meier product limit method. Differences in the survival curves were evaluated with the log-rank test. Survival analyses were done for both overall survival (OS) and progression-free survival (PFS). In OS analysis, deaths were events and being alive was censoring. In PFS analysis, relapses/progressions were events and otherwise were censorings. Cox regression was used for multivariate models that analyzed differences between groups, including patient characteristics with strongest prognostic significance in the univariate analysis (IPI, treatment outcome and GSTM1/T1 genotyping).

RESULTS

The study included 83 DLBCL patients and 89 age and gender matched control subjects. Patient characteristics are detailed in Table (1).

Table (1): DLBCL patient characteristics.

	Total cases N = 83	
Age (year):		
X ± SD	56.95±14.17	
Median	60	
Range	84-18	
LDH (U/L):		
X ± SD	846.86±245.26	
Median	800	
Range	430-1500	
Performance score:	No	%
0	9	10.8
1	26	31.3
2	48	57.8
Stage:		
1	5	6.0
2	28	33.7
3	40	48.2
4	10	12.0
International prognostic index:		
Low	5	6.0
Low intermediate	30	36.1
High intermediate	28	33.7
High	20	24.1
Treatment response:		
CR	71	85.5
PR	12	14.5

X ± SD = Mean ± standard deviation.
LDH = Lactate dehydrogenase.
CR = Complete remission.
PR = Partial remission.

The frequency of GST deletions in 83 Egyptian patients with DLBCL was as follows: GSTM1-null: 62.7% (52/83); GSTT1-null: 22.9% (19/83); GSTM1/T1-double null: 18.1% (15/83). The frequency of GST deletions in 89 Egyptian age and gender matched control subjects was as follows: GSTM1-null: 58.4% (52/89); GSTT1-null: 11.2% (10/89); GSTM1/T1-double null: 6.7% (6/89) (Tables 2,3).

Consequently there was 2.35 fold increase in the risk of DLBCL associated with GSTT1 null genotype (OR=2.35, 95% CI: 1.02-5.40, p=0.04). No significant differences were found in the frequency of GSTM1, genotype in DLBCL cases and controls.

When individuals with both GSTM1 and GSTT1 genes undeleted were considered as a reference, analysis demonstrated comparatively 3 times increased risk for DLBCL in individuals with the GSTM1/T1-double null genotype (OR3.06, 95% CI: 1.04-8.95, p=0.03) (Table 3).

Table (2): Comparison of GSTM1 and GSTT1 genotype frequencies in DLBCL patients and controls (univariate analysis).

Parameter	Studied groups		*p*-value	OR (95% CI)
	Patients N = 83	Controls N = 89		
GSTM1:				
Present	31 (37.3%)	37 (41.6%)	0.57	1.0 (Reference)
Null	52 (62.7%)	52 (58.4%)		1.19 (0.65-2.20)
GSTT1:				
Present	64 (77.1%)	79 (88.8%)	0.04	1.0 (Reference)
Null	19 (22.9%)	10 (11.2%)		2.35 (1.02-5.40)

GST = Glutathione S-transferase gene.
p-value = Probability of error.
$p \leq 0.05$ (Significant).
OR = Odds ratio.
CI = Confidence interval.

Table (3): Combined effects of GSTM1 and GSTT1 null genotypes (multivariate analysis).

		Studied groups		*p*-value	OR (95% CI)
		Patients N = 83	Controls N = 89		
GSTM1:	*GSTT1:*				
Present	Present	27 (32.5%)	33 (37.1%)		1.0 (Reference)
Null	Present	37 (44.6%)	46 (51.7%)	0.96	0.98 (0.50-1.92)
Present	Null	4 (4.8%)	4 (4.5%)	0.78	1.22 (0.28-5.35)
Null	Null	15 (18.1%)	6 (6.7%)	0.03	3.06 (1.04-8.95)

GST = Glutathione S-transferase gene.
p-value = Probability of error.
$p \leq 0.05$ (Significant).
OR = Odds ratio.
CI = Confidence interval.

We found no association between GST genotypes and IPI score. We also found no effect of GST genotypes on the probability to achieve complete remission.

Both Patients showing one GST-null genotype (GSTM1-null genotype or GSTT1-null genotype) and those showing GSTM1/T1-double null genotype significantly had better PFS (p=0.02) and OS (p=0.005) when compared with those showing both or wild GST genes undeleted or wild (Fig. 3A).

Both the IPI score and the treatment outcome proved to be a strong prognostic parameters in our patient group regarding both PFS and OS (p<0.001) (Fig. 2). The probability of PFS and OS in patients with a favorable IPI score (<3) significantly differed according to the GST genotype; patients with one GST deletion and those with GSTM1/T1-double null genotype showed significantly better PFS and OS (p<0.001) (Fig. 3B), while in patients with IPI score ≥3 the OS significantly differed (p=0.001) and the PFS did not differ (p=0.49) according to the GST genotype (Fig. 3C).

Multivariate analysis using Cox regression showed that the presence of at least one GST-null genotype was associated with a 60% reduced risk of relapse/progression (HR=0.40, 95% CI: 0.23-0.69, p=0.001), and 71% reduced risk of death (HR=0.29, 95% CI: 0.16-0.51, p<0.001) (Table 4) i.e. the presence of at least one GST-null genotype tended to have a positive prognostic value independent from both the IPI score and the treatment outcome.

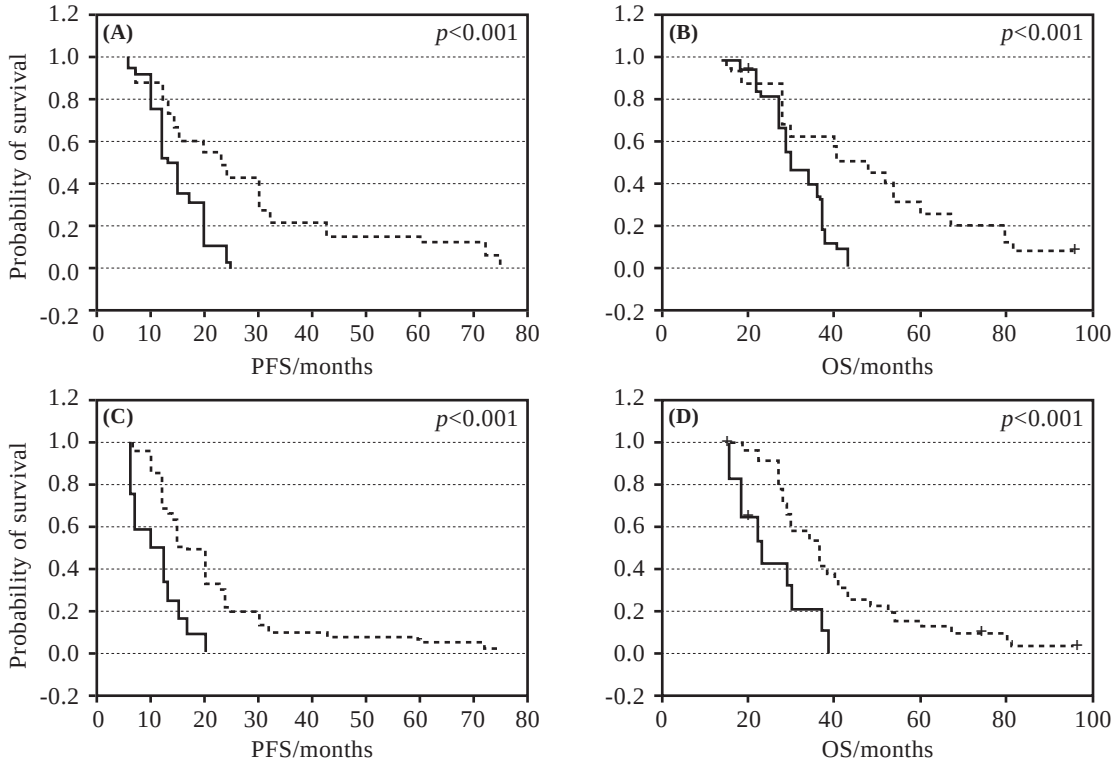

Fig. (2): Progression free survival (PFS) and overall survival (OS) in 83 DLBCL patients according to IPI score (A, B) (---- low IPI, – high IPI, + censored) and treatment outcome (C, D) (---- complete remission, – partial remission, + censored

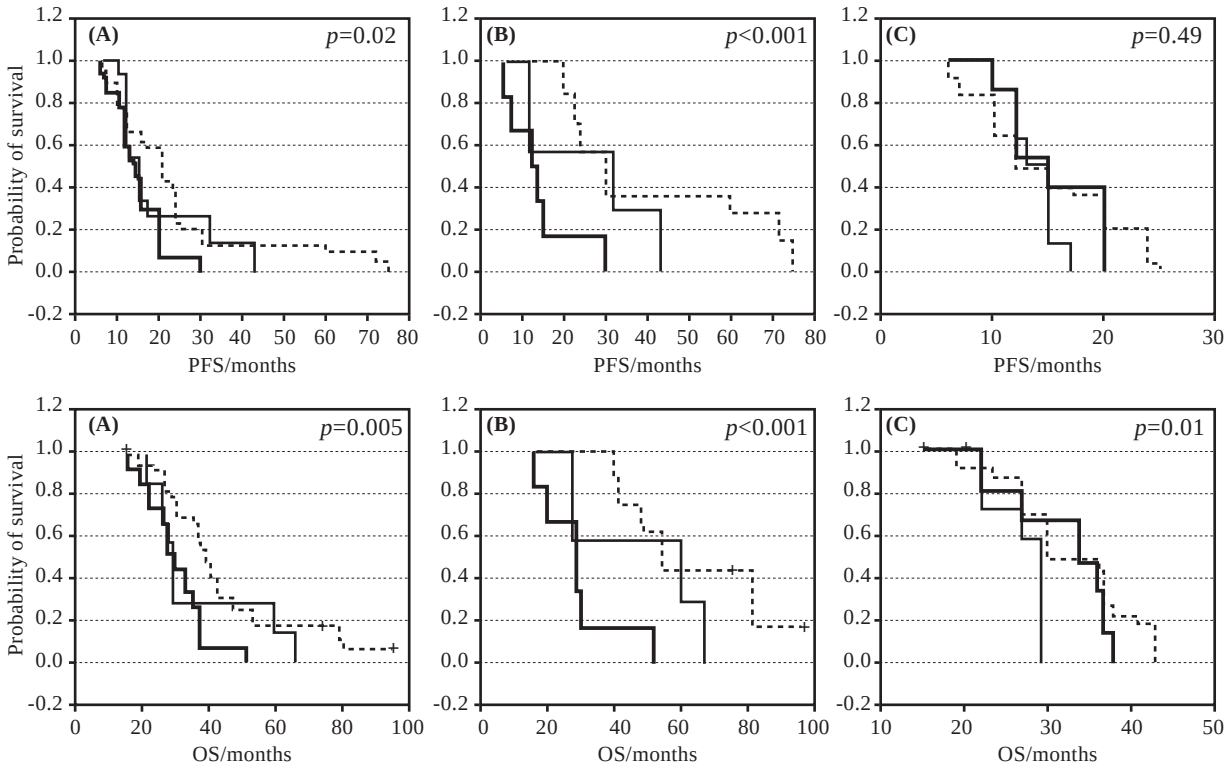

Fig. (3): Progression free survival (PFS) and overall survival (OS) in 83 DLBCL patients (A), in low IPI (<3) patient group (B) and in high IPI (≥3) patient group (C) according to GSTM1 and GSTT1 genotypes (---- one GST deletion, – double-null genotype, **–** wild genotype, + censored).

Table (4): Multivariate Cox regression analysis of variables affecting progression free survival and overall survival in DLBCL patients.

	Hazard ratio	95% CI	*p*-value
A- *Progression free survival:*			
IPI (≥3 vs. <3)	1.98	(1.43 – 2.75)	<0.001
Treatment outcome (PR vs. CR)	2.81	(1.37 – 5.75)	0.005
GST (any deletion vs. wild genotype)	0.40	(0.23 – 0.69)	0.001
B- *Overall survival:*			
IPI (<3 vs. ≥3)	2.29	(1.60 – 3.28)	<0.001
Treatment outcome (PR vs. CR)	3.37	(1.57 – 7.24)	0.002
GST (any deletion vs. wild genotype)	0.29	(0.16 – 0.51)	<0.001

GST = Glutathione S-transferase gene.
IPI = International prognostic index.
p-value = Probability of error.
$p \leq 0.05$ (significant).
OR = Odds ratio.
CI = Confidence interval.

DISCUSSION

GST genotyping was applied in a total of 172 unrelated Egyptian subjects for GSTM1 and GSTT1 genes. Subjects were categorized into 83 DLBCL patients and 89 age and gender matched control subjects. Our hospital (Menofiya University Hospital, Sheben El-Kom, Egypt) is a university hospital serving a rural geographical area. Controls were randomly selected from the same region. This is a farming locality with a known insecticide exposure for inhabitants.

Two studies **[12,13]** investigated GST genotypes on Egyptian subjects. The frequencies of the tested genes in our control Egyptian subjects showed that the frequency of GSTM1-null genotype was 58.4% and that of GSTT1-null genotype was 11.2%. GSTM1-null genotype frequency was a little higher than that reported by Hamdy et al. **[13]** (55.5% among 200 subjects) and was also higher than that reported by Abdel-Rahman et al. **[12]** (44% among 34 subjects). GSTT1-null genotype frequency was a little lower than that reported by Abdel-Rahman et al. **[12]** (15% among 34 subjects) and was considerably lower than that reported by Hamdy et al. **[13]** (29.5% among 200 subjects). This difference could be explained by the older ages of our control subjects. As GST deletions were proved to be associated with a variety of cancers, one can suspect decreasing frequencies of GST deletions with increasing ages of healthy control subjects. The frequencies of GST deletions in our controls were very near to that reported in one study **[7]** performed on Caucasian population; their ages was matched with the ages of our controls. Those were 54% for GSTM1-null genotype and 14% for GSTT1-null genotype (among 205 subjects).

Previous studies on the role of GST polymorphisms in lymphoma risk [NHL overall and/or subtypes including follicular lymphoma (FL), DLBCL & gastric marginal zone lymphoma (GMZL) and Hodgkin's lymphoma (HL)] have given conflicting results, particularly concerning the role of GSTT1-null genotype. Three studies **[14,15,16]** reported no effect of GSTT1-null genotype on lymphoma risk, whereas four **[7,9,8,17]** showed positive associations of this genotype with lymphoma development. Kerridge et al. **[7]** (OR 4.3 for NHL), Al Dayel et al. **[9]** (OR 11.9 for DLBCL), Rollinson et al. **[8]** (OR 9.51 for GMZL) and Hohaus et al. **[18]** (OR 1.9 for HL). Our results also support a significant association between both GSTT1-null and double-null genotypes and an increase in DLBCL susceptibility. GSTT1-null conferred a 2.35-fold increase in DLBCL risk. The double-null genotype conferred a greater risk of 3.06 fold increase in DLBCL (OR was comparable with that reported by Kerridge et al. **[7]**; OR 3.6 and with that reported by Al Dayel et al. **[9]**; OR 3.09). The differences in studies' results could be attributed to racial heterogeneity, controls not selected from the appropriate at risk populations (in Sarmanova et al. **[14]** study) and sample size variability.

As with previous lymphoma studies **[7,8,9,14-17]**, we did not find a significant association between GSTM1-null genotype and DLBCL susceptibility. This together with the finding of GSTT1-null associated DLBCL risk support the importance of GSTT1 gene compared to GSTM1 gene in anti-carcinogenesis though larger studies are required for confirmation.

We found no associations between GST genotypes and IPI score. This is in concordance with Hohaus et al. **[18]** study on FL. However, Cho et al. **[19]** study on DLBCL found that GST1-null genotype was associated with high IPI score. We also, found no association between GST genotypes and treatment outcome. This is

in concordance with Cho et al. **[19]**. However, Hohaus et al. **[18]** reported that Patients with a GSTM1-null genotype had a lower complete remission rate after initial treatment when compared with patients with wild type GSTM1 and the GSTT1 genotype had no effect on the probability to achieve complete remission. Dieckvoss et al. **[20]** found that the presence of at least one GSTM1 allele was of significant beneficial effect on treatment outcome in pediatric patients with non-Hodgkin's lymphoma.

Modulation of prognosis of DLBCL according to genetic polymorphisms has been previously reported in 2 studies **[19,21]**. Both reported that no GST genotype had any impact on OS or event free survival (EFS) **[19]** or on risk of death, risk of relapse or secondary cancer **[21]**. However, in Han et al. study **[21]**, reduced risk of death (HR 0.66) and reduced risk of relapse, secondary cancer or death (HR 0.79) seemed likely. Here, we show that patients with GSTM1/T1-double null genotype or with one GST-null genotype had a significantly better PFS and OS when compared with those with both GST wild genes. Multivariate analysis using Cox regression showed that the presence of at least one GST-null genotype was associated with a 60% reduced risk of relapse/progression and 71% reduced risk of death i.e. the presence of at least one GST-null genotype tended to have a positive prognostic value independent from both IPI score and treatment outcome.

The prognostic impact of GST polymorphisms appears to vary according to the cancer type. Both favorable and unfavorable outcomes have been associated with null genotype of GST enzymes. Our results are in line with previous studies on lymphoma prognosis: Han et al. **[21]** observed that GSTT1-null genotype was associated with a 67% reduced risk of death and a 63% reduced risk of relapse, secondary cancer or death in patients with FL, Hohous et al. **[16]** found that GSTT1-null genotype was more prevalent in patients with low-stage HL than in patients with high-stage HL and Stanulla et al. **[22]** found that GSTT1-null genotype was associated with a reduced risk of relapse in patients with childhood acute lymphoblastic leukemia. On the other hand: Hohaus et al. **[18]** found that GSTT1-null genotype was associated with worse EFS of FL patients. Takanashi et al. **[23]** found that the simultaneous deletion of both GSTM1 and GSTT1 genes was more predictive of early relapse, than any other parameter in childhood precursor-B ALL.

It is assumed that the impact of the GST genotypes on disease control stems from the role of these enzymes in the metabolism of chemotherapeutic drugs. Deletion of GST enzymes could reduce or delay the metabolism of the chemotherapeutic drugs used for DLBCL treatment and may be expected to lead to improved efficacy. However lack of significant association between GST genotypes and treatment outcome could indicate that treatment efficacy may be affected by many other variables such as tumor burden and patient performance.

Conclusion:

Our results have shown a role for the GSTT1-null genotype and the GSTM1/T1-double null genotype as risk factors for DLBCL. The study also, shows that the presence of at least one GST-null genotype tended to have a positive prognostic value for DLBCL patients independent from both the IPI score and the treatment outcome. This might be useful in prediction of DLBCL prognosis and treatment selection. Large-scale studies on rituximab plus CHOP (R-CHOP) treated patients will be needed to either support or modify these findings. A better understanding of the underlying biological mechanisms should be pursued.

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