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GSTM1 Genotype as a risk Modifier of MDR1 C3435T-Induced Risk Susceptibility to Pediatric Acute Lymphoblastic Leukemia

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ABSTRACT

Background: GSTM1 and GSTT1 appear to be associated with a modest increase in the risk of acute lymphoblastic leukemia (ALL). MDR1 C3435T polymorphism was also suggested as a risk factor for childhood ALL; individuals with TT allele have lower expression of P-gp than those with CC genotypes and hence less capable of extruding toxic substances and carcinogens.

Objectives: To investigate the impact of MDR1gene C3435T polymorphism and both GSTM1 and GSTT1 polymorphisms, separately and in combination, on risk susceptibility to childhood ALL.

Patients and methods: The study included 94 children with ALL and 314 apparently health control subjects. Multiplex-Polymerase chain reaction (PCR) was used to evaluate GSTM1 and GSTT1 status while PCR-restriction fragment length polymorphism (PCR-RFLP) was used for the detection of MDR1 C3435T single nucleotide polymorphism.

Results: There was no significant effect of either GSTM1 null or GSTT1 null variant allele or both of them combined on susceptibility to ALL. On the other hand, MDR1 CC, CT and TT genotype frequencies in childhood ALL patients were found to be 78.0%, 17.1% and 4.9%, respectively vs. 91.2%, 8.8% and 0 % in the control group (p=0.016). MDR1 gene C3435T homozygote and heterozygote have a 2.9 fold increased risk to develop ALL (OR=2.918, 95% CI:1.193-7.137,). There is a significant synergistic association between GSTM1-null allele and mutant MDR genotype homozygous TT or heterozygous allele CT on susceptibility to ALL with a 3.672 fold increased risk (p-value. 0.032 OR=3.672, 95% CI:1.059-12.733), however the presence of GSTM1 abolished the effect of mutant MDR1 allele on risk susceptibility to ALL (p-value. 0.193). In conclusion, the increased risk to develop pediatric ALL associated with MDR1 gene C3435T homozygote and heterozygote is further potentiated by the presence of GSTM1 null and abolished by the presence of GSTM1wild. Molecular genetic analysis is still required to understand genotype-genotype interaction and to clarify genotype-phenotype relation and their reflection on disease risk.

Key Words: *Glutathione s-transferase – GSTM1 – GSTT – MDR1 – Combined genotype polymorphisms – Acute lymphoblastic leukemia.*

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is a frequent malignancy affecting both children and adults. Despite much investigation, the causes are not yet fully understood. Like many other cancers, acute leukemia is considered to be a complex disease, which is determined by a combination of genetic and environmental factors **[1,2]**. There is increasing evidence that predisposition to acute leukemia is associated with exposure to chemicals such as benzene and chemotherapeutic agents **[3,4]**. Glutathione S-transferases (GSTs) are a family of cytosolic enzymes involved in the detoxification of various exogenous as well as endogenous reactive species **[5,6]**. GSTs function as dimers by catalyzing the conjugation of mutagenic electrophilic substrates to glutathione. In humans, 4 major subfamilies of GSTs can be distinguished and are designated as GSTα, GSTμ, GST0, and GSTπ. Within the GSTμ subfamily, the gene coding for GSTM1 exhibits a deletion polymorphism which in case of homozygozity (GSTM1 null) leads to absence of phenotypic enzyme activity **[7]**. Similar mechanism is described for GSTT1 within the GST0 subfamily **[8]**. Variant GST alleles have been identified within the general population. The most extensively studied variant GSTs include two GST deletion alleles namely GSTM1*0/*0 and GSTT1*0/*0 **[9]**. The functional consequences of the GSTM1 and GSTT1 (*0/*0) genotypes are obvious in terms of enzyme activity; gene deletion results in loss of conjugation potential. Molecular epidemio-

logical studies indicate that individuals lacking the genes GSTM1 and GSTT1 are more likely to develop cancer than those having these genes **[6]**. Based upon this meta-analysis of 30 case-control studies, GSTM1 and GSTT1 appear to be associated with a modest increase in the risk of acute lymphoblastic leukemia (ALL). It is conceivable that GSTM1 and GSTT1 null genotypes may thus play a role in leukemogenesis. The pooled analysis of both GSTM1 and GSTT1 null genotypes produced a similar risk estimate **[10]**. Thus, some studies indicate that GST polymorphisms are associated with ALL; however, this association is not accepted across all observational studies. Discrepancies may be partially attributed to failure to consider gene combinations or interactions with environmental factors. Greater understanding of the numerous factors affecting GST expression and activity, accompanied by more incisive genetic analysis, may reveal further connections between GST genotypes and ALL risk **[11]**.

The multi-drug resistance 1 gene (MDR1) which belongs to the family of ABC transporter proteins, encodes P-glycoprotein (P-gp). P-gp is a membrane-associated protein that acts as an ATP-dependent pump involved in the membrane transport of various substrates including toxic xenobiotics **[12-15]** and it thus has a protective function in various cells and tissues/organs **[16]**. Several polymorphisms of this gene have been characterized. Several studies suggested that MDR1 C3435T polymorphism was a risk factor for childhood ALL. Carriers of the TT genotype are more at risk of developing ALL than other individuals, whereas CC genotype carriers are supposed to have worse prognosis **[17-20]**.

To the best of our knowledge, there is no study addressing the potential synergistic effect of GSTM1 and/or GSTT1 Null and MDR1 c>T polymorphism risk susceptibility to pediatric ALL. In this study we examined GSTM1 and GSTT1status and C3435T polymorphism in 94 pediatric ALL cases and 314 apparently healthy controls to verify if any of their impact on risk susceptibility to ALL separately and in combination.

PATIENTS AND METHODS

The study included 94 newly diagnosed Pediatric ALL patients who presented to the Pediatric Oncology department, NCI, Cairo University and 314 apparently healthy controls blood bank donors. We chose adults to avoid ethical concerns of using children as control taking in consideration that genotype is not affected by age. Diagnosis was performed according to clinical, morphological, cytochemical and immunophenotypic examination. The IRB of the NCI, Cairo University approved the study and written informed consent was obtained from all participants or their guardians.

The criteria for inclusion in patient's group were:

1- Egyptians origin residing in Egypt area as judged by their names, languages and places of birth.

2- Availability of biological material.

The recruited patients comprised 58 males and 36 females between the ages of 1.5 and 18 years with a median of 6 years.

The criteria for inclusion in the control group were:

1- Anonymous, healthy, and unrelated individuals.

2- Egyptians origin residing in Egypt area as judged by their language and place of birth.

Genotyping:

DNA isolation: DNA was isolated from peripheral blood at diagnosis using Qiagen column Kit (USA) according to the manufacturer's recommendations. DNA concentration was determined by measuring the optical density at 260 nm and the purity of the nucleic acid (the absence of proteins contamination) was determined by the ratio of absorbance at 260 nm to the absorbance at 280 nm. The ratio of 260/280 should be 1.7-2.0 **[21]**.

Genotyping for MDR1 C2334T was performed by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method and GSTM1 and GSTT1 were performed by multiplex PCR with the housekeeping gene B-globin as internal control.

GSTM1 polymorphism:

The polymorphic deletion of the GSTM1 gene was genotyped using the multiplex PCR of GSTM1 Primers with ß-globin housekeeping gene used as internal control as described by

[22]. PCR was performed in 20 µL reaction mix containing 20 ng of genomic DNA, 0.5 µmol/L of each primer, 200 µmol/L of each dNTPs, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, and 0.5 U of ampliTaq DNA polymerase (Hoffman-LaRoche, Branchburg, NJ). After initial denaturation for 10 minutes at 95°C, the PCR was performed for 35 cycles of 1 minute at 94°C, 1 minute at 59°C, and 2 minute at 72°C. The last elongation step was extended to 7 minutes. Negative and positive control samples were included in each amplification series. The amplicon was analyzed by electrophoresis on a 3-4% agarose gel, at 100 volt for 30 min. The presence of GSTM1 allele, identified by a 219-bp fragment indicates wild type while its absence indicates complete deletion (null genotype) **[23]**.

GSTT1 Polymorphism:

The polymorphic deletion of the GSTT1 gene was genotyped using the multiplex PCR of GSTT1 Primers and ß-globin housekeeping gene as internal control **[22]**. The amplification reaction was performed in 20 µL reaction mix, containing 20 ng of genomic DNA, 0.5 µmol/L of each primer, 200 µmol/L of each dNTPs, 2.0 mmol/L $MgCl_2$, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, and 0.5 U ampliTaq DNA polymerase (Hoffman-LaRoche). After initial denaturation for 10 minutes at 95°C, the PCR was performed for 35 cycles of 1 minute at 95°C, 1 minute at 59°C, and 2 minute at 72°C. The last elongation step was extended to 7 minutes. Negative and positive control samples were included in each amplification series. The amplicon was analyzed by electrophoresis on a 1.5% agarose gel, at 100 volt for 30 min. The presences of GSTT1 alleles, identified by a 480-bp fragment indicate wild type while its complete deletion indicates null genotype **[23]**.

MDR1:

MDR1 C3435T mutation was determined using PCR-RFLP assay **[24]**. The PCR products were digested at 37 Cº using 4 units Sau3AI restriction endonuclease (New England BioLabs, UK). The restriction fragments obtained were separated by electrophoresis on a 3% agarose gel for 45 min at 140V and analyzed after staining with ethidium bromide under ultraviolet light. The 3435-C (wild type) allele can be detected by the presence of two fragments, which are 158 bp and 39 bp long. The presence of 3435-T (Mutant type) allele results in uncut amplified segment of 197 bp; the presence of a heterozygous genotype results in the presence of all three bands. The primer design was based on published sequences (Table 1).

Table (1): The oligonucleotide primer pairs used for amplification of the candidate genes.

Gene/ primer	Primer sequence	Reference
GSTM1 forward	GAA CTC CCT GAA AAG CTA AAG C	23
GSTM1 reverse	GTT GGG CTC AAA TAT ACG GTG G	
GSTT1 forward	TTC CTT ACT GGT CCT CAC ATC TC	23
GSTT1 reverse	TCA CCG GAT CAT GGC CAG CA	
ß-Globin forward	ACA CAA CTG TGT TCA CTA GC	23
ß-Globin reverse	CAA CTT CAT CCA CGT TCA CC	
MDR1 Forward	TGTTTTCAGCTGCTTGATGG	24
MDR1 reverse	AAGGCATGTATGTTGGCCTC	

Statistical analysis:

Statistical analysis was performed using SPSS 15.0 (Statistical Package for the Social Science). The level of significance was calculated by Fisher's exact test. Odds Ratio (OR) was used to measure the strength of association between the tested genotypes and ALL risk. Crude ORs are given with 95% Confidence interval (CI). All of the statistical tests were based on two-tailed probability.

RESULTS

Glutathione S transferase:

The frequencies of the GSTM1 wild allele was 46/94 (49%) among patients compared to 141/314 (44.9%) among controls. The frequency of the GSTM1 deleted (Null) allele was 47 (51%) among patients compared to 173 (55.1%) among controls; the difference was found to be statistically insignificant (p-value=0.553). No significant association was observed between GSTM1 genotype and ALL development.

The frequency of the GSTT1 present allele was 76 (82.6%) among patients compared to

257 (86%) among controls. No significant association was observed between GSTT1 genotype and ALL development. The frequency of the GSTT1 deleted (null) allele was 16 (17.4%) among patients compared to 42 (14%) among controls; the difference was found to be statistically insignificant (*p*-value=0.430).

The GSTM1 and T1 genotypes were double deleted in 4 ALL patients (4.4%) cases compared to 25 normal controls (8.4%); the difference was found to be statistically insignificant, *p*-value=0.069.

The MDR C3435T genotypes distribution among the study samples of 80 childhoods ALL patients in comparison to 91 normal healthy controls. The frequency of the MDR C/C, C/T and T/T alleles were 64 (78 %), 14 (17.1%) and 4 (4.9%) respectively among patients compared to 83 (91.2%), 8 (8.8%) and 0 (0%) among controls as shown in (Table 2). MDR 3435 CT Heterozygous and TT Homozygous genotype polymorphism is significantly associated with childhood ALL (*p*-value=0.016) which result in increased risk of ALL by 2.91 folds. Also, T allele was significantly high in Children All (13.4%) as compared to control (4.4%), *p*=0.035. The risk to ALL increased in ALL patients (OR= 3.37).

When we studied the frequency of both MDR 3436 CT/TT genotypes and null GSTM1 genotype we found a significantly higher frequency in ALL patients than controls (*p*=0.032) (Table 3). These combined genotypes showed an increased risk of ALL by 3.67-folds.

Table (2): MDR1 C3435T genotyping and allele frequency among ALL cases and Controls.

MDR1 C3435T	C/C		C/T		T/T		C/T+T/T		C allele	T allele
	No	%	No	%	No	%	No	%	%	%
Control: n=91	83	91.2	8	8.8	0	0	8	8.8	95.6	4.4
ALL: n=82	64	78	14	17.1	4	4.9	18	22.0	86.6	13.4
p			0.078				0.016*		0.035	
OR			2.270				2.91		3.37	
95% CI			0.897-5.740				1.193-7.137		1.029-11.038	

Table (3): MDR1 C3435T combined to GSTM1 or GSTT1deleted genotypes among ALL and control groups.

Gene	Genotype	ALL (n=56)		Controls (n=67)		*p*-value	OR	95% CI
		N	%	N	%			
MDR1C3435T and GSTM1 null	CT+TT+M1null	10	23.8%	4	.8	0.032*	.672	1.059-12.73
	CC+M1present	32	76.2%	47	92.2			
	Total	42	100	51	100			
MDR1C3435Tand GSTT1null	CT+TT+T1 null	6	42.9	2	12.5	0.101	5.250	0.850 - 32.430
	CC+T1present	8	57.1	14	87.5			
	Total	14	100	16	100			
MDR1C3435Tand GSTM1 present	CT+TT+M1present	8	20.5	4	10	0.193	2.323	0.638 - 8.461
	CC+M1 present	31	79.5	36	90			
	Total	39	100	40	100			
MDR1C3435Tand GSTT1 present	CT+TT+T1 present	12	17.6	6	8	0.082	2.46	0.870 - 6.982
	CC+T1 present	56	82.4	69	92			
	Total	68	100	75	100			

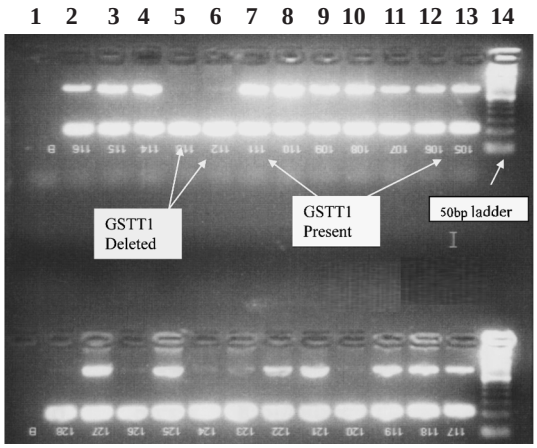

Fig. (1): GSTT1 and B-Globin in multiplex-PCR Lane 1: No amplification. Lane 2-4, 7-13: GSTT1Present (480 bp) + B-Globin (110 bp). Lane 5, 6: GSTT1 Deleted (B-Globin110 bp). Lane 14:50 bp marker.

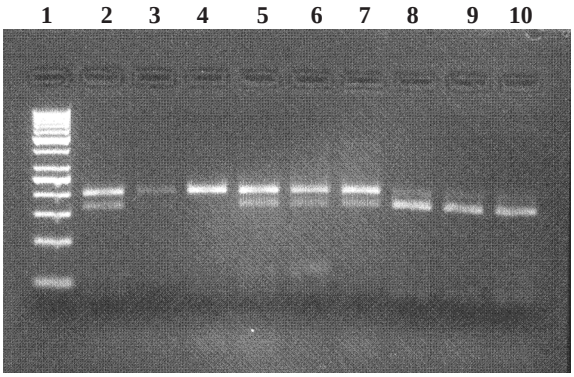

Fig. (2): MDR1 C3435T polymorphism after Sau3AI Digestion Lane: 50 bp DNA ladder. Lane 2: Heterozygous genotype. Lane 3 & 4: Homozygous. Lane 5-7: Heterozygous. Lane 8-10 Wilde type.

DISCUSSION

In our study, GSTM1 null and GSTT1 null genotype has no effect on the risk of ALL. Our findings are in agreement with Chen et al. **[25]**. In their study, the GSTM1 null genotype was detected in 55.2% of white colored children with ALL and 53.5% of normal controls, and the GSTT1 null genotype was found in 14.1% of white colored children with ALL and 15.0% of their controls. In our study, deletions in GSTM1 and GSTT1 genotypes either separately or in combination were not found to be significantly associated with an increased risk of acute leukemia. Thus, combined GSTM1 and GSTT1 genotypes did not show any synergistic effect on pediatric ALL susceptibility. This is contrary to many studies **[9,10,26-28]** which reported that individuals with null GSTM1/GSTT1 genotypes may have an impaired ability to detoxify carcinogens, thus, carrying an increased risk of developing cancer. Arruda et al. **[2]** observed a 4.7-fold (95% CI: 2.1-11.0) and 2.3-fold (95% CI: 1.0-5.2) increased risk of acute myeloid leukemia with the GSTM1 and GSTT1 null genotypes, respectively, and a 6.6-fold (95% CI: 2.4-7.9) increased risk with the combined null genotype. Although many studies have shown significant association of GST polymorphisms with acute leukemia, others have shown contradictory results **[10]**. This may be attributed to many factors like different ethnic group and exposure to different types of carcinogens in different environments which means different gene-environment interaction. Also sample size may affect the results of the study. Therefore, studies with large sample sizes will reflect much better the association of genotypes and cancer risk since they provide statistical power. Meta-analyses studies have indicated statistically significant but small increases in risk for specific genotypes, while many studies have been negative **[10]**. However, the genetic analysis used in most of these studies has been limited, especially by the failure to discriminate between heterozygous and homozygous deletion genotypes (gene dose). It is also well known that humans express a large number of different GSTs with overlapping substrate specificities, and the effects of polymorphisms (including gene deletions) affecting one GST may be masked by the activity of others. On the other hand, GST activity is highly variable among individuals, but genetic factors may account for only a fraction of this variability **[29,30]**. Factors such as diet **[31]**, environmental chemical exposures **[32]**, age **[33]**, or gender **[34]**; that remain only poorly understood; may be more important determinants. Nevertheless, our understanding of human GST polymorphisms is still incomplete which hardens any interpretation of contradictions in literature relevant to clinical aspects.

In our work, MDR1 C3435T gene polymorphism showed significant impact on the risk susceptibility to pediatric ALL. In the present study, MDR1 C3435T heterozygous (CT) and homozygous (TT) genotypes was associated with 2.91-fold increased risk of childhood ALL while MDR1 3435T allele frequency was associated with 3.37 fold increased risk. The risk is in line with previous findings showing that patients with MDR1 C3435T TT-allele might

be at a higher risk of development of ALL than those with C3435T CC-allele **[17-20]**.

The potential mechanism of the interaction of MDR1 polymorphisms with GSTM1 and GSTT1 in modifying risk susceptibility to childhood ALL has not yet been satisfactorily described. In the present study, we have compared the frequency of MDR1 C3435T combined with GSTM1 and/or T1 polymorphism in childhood ALL patients and healthy controls in an attempt to identify a possible impact of combined gene variation on the susceptibility to ALL. The MDR 3436TT genotype combined to GSTM1 null genotype showed a significantly higher frequency in ALL patients than controls (p=0.032). With the complex metabolic pathways of xenobiotics, synergistic or antagonistic interactions between genotypes are of great interest as they affect phenotypic features In our work, MDR T allele carriers when associated with deleted GSTM1 genotype, showed significantly higher risk than CC genotype individuals. We found a 3.67-fold increased risk of ALL when MDR C3436T (CT/TT) genotypes are combined with GSTM1 null allele compared to 2.91 fold increased risk of All in MDR genotype alone. On the other hand, the presence of both GSTM1 and GSTT1 abolish the risk effect of the variant allele of MDR1 C3435T gene polymorphism. This means that when the GST M1 and T1 enzyme activity are normal they can overcome the decreased efflux due to the presence of variant allele of MDR1 C3435T polymorphism and detoxify carcinogens.

In contrast to our findings, MDR1 polymorphisms have not been shown to be associated with overall risk of ALL in previous studies **[35,36]**. However, in one of these studies, the patient sample size was 44 **[35]**. The association of MDR1 C3435T gene polymorphism with incidence of different diseases is yet controversial. However, the genotype TT was shown to be associated with more than two-fold lower duodenal P-gp expression levels compared with CC genotype reducing the rate of efflux of carcinogens. There are several hypotheses regarding the influence of this polymorphism on phenotypically revealed features. One hypothesis assumes a change of the substrate's affinity to the P-gp transporter **[37]**. These authors showed that the polymorphism alters the substrate specificity in mammalian membrane transport protein affecting the timing of co-translational folding and may result in altered function. Other authors suggested that MDR1 C3435T is a silent polymorphism which leads to a more unstable mRNA and consequently, lowering overall activity of the variant allele **[38]**.

The very large MDR1 gene includes 28 exons and is highly polymorphic which makes it difficult to identify causal polymorphisms. In addition, linkage patterns and allele frequencies in MDR1 are highly variable between different ethnic groups and thus between the studied populations. Hence, case-control studies with assessment of multiple polymorphisms in parallel with P-glycoprotein activity, mRNA and protein level measurements will be required to understand MDR1 genotype-phenotype relation.

In conclusion, the present study indicated that the studied GSTM1 and GSTT1 polymorphisms are not associated with risk of ALL, whereas MDR polymorphism was found to increase the risk of childhood ALL.

To our knowledge, this is the first study showing combination of GST polymorphisms affecting detoxification reaction and MDR genotypes controlling the efflux of carcinogens in association with childhood ALL. This gene interaction synergistically increases the susceptibility to childhood ALL in homozygote or heterozygote MDR1 C3435T variant type carriers associated with null GSTM1 polymorphism while the present GSTM1 and T abolish this risk effect. The present results stress the importance of molecular genetic analysis of combined genotypes with different role in carcinogenesis in stratifying individuals at increased risk of ALL.

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