

**INFLUENCE OF GLUTATHIONE – S – TRANSFERASE (GSTT1 AND GSTM1)
POLYMORPHISM ON BASELINE MICRONUCLEI FREQUENCY IN PERIPHERAL
BLOOD LYMPHOCYTES**

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We have analyzed impact of polymorphism in GSTT1 and GSTM1 genes on the micronuclei (MN) frequency in peripheral blood lymphocytes (PBLs). A total 134 women from central Serbia were enrolled in the study. Polymorphisms of GST genes were genotyped by performing a multiplex polymerase chain reaction (PCR) and cytokinesis block micronucleus (CBMN) test was used to assess MN frequency. GSTT1 null and GSTM1 null genotype carriers had higher MN frequencies as compared to positive counterparts but without statistical significance. Carriers of dual GSTT1/GSTM1 null genotypes had significantly higher MN frequency than positive/positive, positive/null and null/positive. Smokers and women >45 years old with GSTT1 null genotype and GSTT1null/GSTM1null genotypes have statistically higher MN frequency than positive counterparts. Results suggest possible influence of dual null genotypes of GSTT1/GSTM1 on the baseline MN frequency, as well influence on the level of MN in smokers and in women age >45 years. GSTT1 null genotype may have the potential to influence the baseline MN frequency in PBLs of smokers, as well as in women age >45 years.

Key words: GSTT1, GSTM1, micronuclei, peripheral blood lymphocytes, polymorphism

INTRODUCTION

Interindividual variability in the frequency of micronuclei (MN) in human peripheral blood lymphocytes has been revealed (KOPJAR *et al.*, 2010; MILOŠEVIĆ-DJORDJEVIĆ *et al.*, 2011).

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The variability in MN frequency in lymphocytes has been associated with different factors, including demographic and lifestyle factors. Nevertheless, during the last decades the associations between the level of genetic damages in lymphocytes of exposed/unexposed subjects and genetic constitution have been extensively studied (SCHRODER *et al.*, 1995; FALCK *et al.*, 1999; ISHIKAWA *et al.*, 2004; TEIXEIRA *et al.*, 2004; ZIJNO *et al.*, 2006; KUMAR *et al.*, 2011; SINGH *et al.*, 2012).

Among different genetic polymorphisms, a great attention has been focused on the genes involved in detoxification. Glutathione S-transferase (GSTs) as enzymes that belong to the Phase II has been of interest in many studies. They are involved in detoxification of xenobiotics by conjugating a variety of chemicals with reduced glutathione (GSH) (PALMA *et al.*, 2007). Based on amino acid sequence identity, human cytosolic GSTs are divided into seven subfamilies: alpha, mu, pi, theta, zeta, omega, and sigma (WU and DONG, 2012). Human GSTs classes mu and theta are very often studied in human populations. Mu class genes are located on chromosome 1p13.3 and genes of this class are situated in tandem in a 20 kb (XU *et al.*, 1998). Theta class includes two genes GSTT1 and GSTT2 and they are located on the chromosome 22, precisely to the subband 22q11.2 (TAN *et al.*, 1995; WEBB *et al.*, 1996); GSTT1 enzyme has dual, detoxifying and activating properties (DHILLON *et al.*, 2011). Both human cytosolic GSTT1 and GSTM1 exhibit deletion polymorphisms that in homozygous individual results in lack of enzyme activity and it is presumed that individual with null genotype have reduced capacity of detoxification. The association between this polymorphism and disease risk has been examined in number of studies (PEREZ-PASTENE *et al.*, 2007; ROHR *et al.*, 2008; SAFARINEJAD *et al.*, 2010; AMER *et al.*, 2011; ROCHA *et al.*, 2011).

Micronucleus assay is one of the most applied test in cytogenetic studies. Micronuclei are small nuclei clearly separated from the main nuclei containing whole chromosome or fragments/chromatids that lag behind in anaphase and left out of daughter nuclei during the cell division. Micronuclei are useful biomarker of genotoxicity, detecting both *clastogenic* and *aneugenic* compounds. The analysis of micronuclei (MN) in peripheral lymphocytes is a useful biomarker of genetic damages. Lymphocytes are long lived cells that during life span circulations through different organs and tissues accumulate DNA damages; therefore lymphocytes are very often used in biomonitoring studies for assessing chromosomal damages. So far, a great number of studies revealed positive correlation between different pathological conditions and MN frequency in peripheral blood lymphocytes (MILOŠEVIĆ-DJORDJEVIĆ *et al.*, 2011; TRKOVÁ *et al.*, 2000; VRNDIĆ *et al.*, 2013). Moreover, authors suggested that MN in peripheral blood lymphocytes of healthy individuals is suitable cytogenetic biomarker for cancer (BONASSI *et al.*, 2007) or cardiovascular diseases (MURGIA *et al.*, 2007).

Here, we evaluated the possible influence of the genotype (in particular, polymorphism in two genes GSTT1 and GSTM1) on baseline MN frequency in peripheral blood lymphocytes women from central Serbia.

MATERIALS AND METHODS

Study subjects

The study has been approved by Ethics Committee of the Clinic of Kragujevac (N^o 2577) and Faculty of Medicine University of Niš (01-5518-1). The study population enrolled 134 women from central Serbia. For all participant informed consent and questionnaire, covering

standard demographic, occupational and medical questions, was obtained at the time of collecting the blood.

Micronuclei assay

The CBMN assay described by FENECH and MORLEY (1985) was carried out for determination the basal genetic damages. In brief, aliquots 0.5 ml of heparinized whole blood was added into 5 ml of complete medium for lymphocytes cultivation PBMax Karyotyping (Invitrogen, California, USA). For each woman double culture were set up and incubated for 72h at 37°C. Forty four hour of beginning the cultivation cytochalasin B was added to the culture, in the final concentration of 4 µg/ml. After another 28h the cells were harvested. The cells were two times treated with cold hypotonic solution, and fixed three times with a fresh prepared fixative (methanol:glacial acetic acid = 3:1). At the end, air dried slides were stained in 2% Giemsa solution for 12 minutes (Alfapanon, Novi Sad, Serbia).

The scoring was performed using a light microscope (Nikon E50i) at 400 x magnification. For each woman micronuclei frequency were scored in 1000 binucleated cells following the criteria described by FENECH (2007). Nuclear division index was calculated using the formula: $NDI = ((1 \times M1 + (2 \times M2) + (3 \times M3) + (4 \times M4))/N)$, where M1-M4 are the number of cells with 1 to 4 nuclei, and N is the number of the scored cells (FENECH, 2000).

DNA isolation and genotypic analysis

Genomic DNA was isolated from 350 µl of whole blood using the EZ1 DNA Blood 350 µl Kit (Qiagen, Hilden, Germany) and BioRobot EZ1, following the manufacturer's instructions.

The analysis of GSTT1 and GSTM1 genotypes was performed simultaneously by a multiplex polymerase chain reaction (PCR), as described ABDEL- RAHMAN *et al.* (1996) with little modification. Briefly, in 50 µl PCR reaction mix containing 30 pmol of each GSTT1 primers (Invitrogen, California, USA) GSTM1 primers (Invitrogen, California, USA) and CYP1A1 (Invitrogen, California, USA), 200 µM of each deoxynucleotide triphosphate (Invitrogen, California, USA), 1.5mM of MgCl₂, 1X PCR buffer, 2U of Taq polymerase (Invitrogen, California, USA), 5% and dimethyl sulfoxide (DMSO) and 5% glycerol, 50 ng of template DNA was amplified. 35 cycles of denaturation at 94° C for 2 minutes, annealing for 1 minute at 58° C and extension at 72° C for 1 minute, followed by a final extension step for 10 minutes at 72° C, were performed, after initiative denaturation at 94° C for 5 minutes.

PCR products were separated and analyzed on 2% agarose gel stained with SYBER Safe DNA gel stain (Invitrogen, California, USA). The presence of 480 bp band corresponds to GSTT1 positive genotype while 215 bp band corresponds to GSTM1 positive genotype. The band of internal control CYP1A1 (312 bp) was always present.

Statistical analysis

All results are shown as mean ± standard deviation (S.D.). Differences in MN frequencies between the groups (positive genotype vs. negative genotype) were analyzed by using the appropriate parametric/nonparametric test. Additionally, multiple linear regression analyses was performed for analyzing the influence of independent variables (genotypes) on MN frequency and NDI values. Probability less than 0.05, 0.01 and 0.001 were set up as significant.

RESULTS

The general demographic, habitual, medicinal and reproductive characteristics, as well the distribution of GSTT1 and GSTM1 genotypes of study subjects are shown in Table 1.

Table 1. General characteristics of the study population

Number of subjects	134
Age (mean± S.D, range)	43.72±11.07
Smoking habit	
Smokers	73
Years of smoking (mean ± S.D, range)	16.48±8.50 (2.5 - 40)
Cigarettes per day (mean ± S.D, range)	15.03±7.51 (1 - 40)
Nonsmokers	61
Reproductive history	
Miscarriages (range)	36 (1- 4)
Abortions (range)	64(1-10)
GSTT1	
positive	86
null	48
GSTM1	
positive	43
null	91
GSTT1/GSTM1	
positive/positive	26
positive/null	60
null/positive	17
null/null	31

As is presented in Table 2 carriers of GSTT1 null and GSTM1 null genotype had higher MN frequencies as compared to GSTT1 positive and GSTM1 positive genotype (13.42±5.23/1000 BN cells vs. 11.80± 4.53/1000 BN cells and 12.79±5.09/1000 BN cells vs. 11.51±4.16/1000 BN cells, respectively), but without statistical significance. Further, women with dual GSTT1/GSTM1 null genotypes had significantly higher MN frequency in peripheral blood lymphocytes than women with GSTT1positive/GSTM1positive, GSTT1positive/GSTM1 null and GSTT1null/GSTM1positive (14.55±4.99/1000 BN cells vs. 11.61±3.45/1000 BN cells, 11.88±4.95/1000 BN cells, 11.35±5.17/1000 BN cells, respectively).

According to the smoking habit in both groups, smokers and nonsmokers, women with GSTT1 or GSTM1null genotype had higher MN frequency as compared to the women with positive genotypes, but with statistical significance only for GSTT1 in smokers. Women with GSTT1null/GSTM1null genotypes had significantly higher MN frequency among smokers compared to GSTT1positive/GSTM1positive and GSTT1positive/GSTM1null counterparts. Similarly, in nonsmokers group we revealed the highest MN frequency in lymphocytes of GSTT1null/GSTM1null genotypes carriers, but neither of analyzed genotype combinations significantly modulates MN frequency.

Table 2. Mean MN frequencies in study subject according to the GSTT1 and GSTM1 genotype status

	Genotypes							
	GSTT1		GSTM1		GSTT1/GSTM1			null/null
	positive	null	positive	null	Positive /positive	Positive /null	Null /positive	
Total (134)	11.80±4.53	13.42±5.23	11.51±4.16	12.79±5.09	11.61±3.45	11.88±4.95	11.35±5.17	14.55±4.99 ^a
Smoking habit								
Smokers (73)	11.46±3.84	14.20±5.27 ^b	11.56±3.98	12.83±4.79	11.50±3.08	11.44±4.29	11.67±5.45	15.62±4.74 ^c
Nonsmokers (61)	12.24±5.29	12.56±5.18	11.44±4.51	12.74±5.48	11.80±4.16	12.39±5.70	11.00±5.18	13.40±5.15
Age								
≤45 (74)	10.63±4.25	10.52±4.91	9.96±3.67	10.92±4.78	10.43±3.37	10.70±4.58	9.36±4.10	11.58±5.50
>45 (60)	13.51±4.43	16.08±4.02 ^d	13.67±3.91	14.98±4.60	13.00±3.13	13.78±5.02	15.00±5.21	16.42±3.67 ^e

^a statistically significant differences in MN frequencies between GSTT1null/GSTM1null and GSTT1positive/GSTM1positive, GSTT1positive/GSTM1null, GSTT1null/GSTM1positive

^b statistically significant differences in MN frequencies between GSTT1null and GSTT1positive

^c statistically significant differences in MN frequencies between GSTT1null/GSTM1null and GSTT1positive/GSTM1positive, as well between GSTT1null/GSTM1null and GSTT1positive/GSTM1null

^d statistically significant differences in MN frequencies between GSTT1null and GSTT1positive

^e statistically significant differences in MN frequencies between GSTT1null/GSTM1null and GSTT1positive/GSTM1positive

Considering the age, GSTT1 null as well GSTT1null/GSTM1null women aged >45 years showed statistically higher MN frequency than positive counterparts. Women with GSTM1 null genotype had higher MN frequency in both age groups, but as for either analyzed combinations in < 45 years old group, the differences in MN frequencies did not reach statistical significance.

The mean NDI values in analyzed women according to the genotype status are presented in Table 3. Our results show that neither of analyzed genotypes, alone or in combinations, influenced the NDI values. Statistical significant differences were only noticed in NDI values between women age ≤45 years with GSTT1null/GSTM1null genotypes and GSTT1positive/GSTM1null. Similar, statistically different NDI values has been noticed in women age >45 years caring GSTT1positive/GSTM1null combination compared to positive/positive and null/null compared to positive/null.

Overall, multiple regression analysis showed that MN frequency and NDI values were not affected by GSTT1 null and GSTM1 null genotype (Table 4).

Table 3. Mean NDI values in study subject according to the GSTT1 and GSTM1 genotype status

	Genotypes							
	GSTT1		GSTM1		GSTT1/GSTM1			null/null
	positive	null	positive	null	Positive /positive	Positive /null	Null /positive	
Total (134)	1.69±0.11	1.69±0.13	1.69±0.12	1.69±0.12	1.68±0.12	1.70±0.10	1.69±0.11	1.68±0.14
Smoking habit								
Smokers (73)	1.68±0.13	1.66±0.16	1.67±0.14	1.68±0.14	1.67±0.15	1.69±0.12	1.68±0.12	1.65±0.17
Nonsmokers (61)	1.71±0.07	1.71±0.08	1.71±0.08	1.71±0.08	1.71±0.07	1.71±0.08	1.71±0.09	1.72±0.08
Age								
≤45 (74)	1.69±0.12	1.73±0.10	1.71±0.12	1.69±0.12	1.71±0.15	1.68±0.12	1.72±0.09	1.74±0.10 ^a
>45 (60)	1.70±0.08	1.65±0.14	1.65±0.10	1.69±0.12	1.65±0.09	1.73±0.07 ^b	1.65±0.13	1.65±0.15 ^c

^a statistically significant differences in NDI between GSTT1null/GSTM1null genotypes and GSTT1positive/GSTM1null.

^b statistically significant differences in NDI between GSTT1positive/GSTM1null and GSTT1positive/GSTM1positive

^c statistically significant differences in NDI between GSTT1null/GSTM1null compared to GSTT1positive/GSTM1null.

Table 4. Results of multiple regression analyses for micronucleus frequency including GSTs status

		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
		MN	Constant	13.902		
	GSTT1	-1.685	0.860	-0.168	-1.960	0.052
	GSTM1	-1.372	0.883	-0.133	-1.553	0.123
NDI	Constant	1.690	0.019		90.855	0.000
	GSTT1	0.007	0.021	0.028	0.323	0.747
	GSTM1	-0.005	0.22	-0.021	-0.244	0.808

DISCUSSION

Until today, a number of studies considering the association between cytogenetic biomarkers in exposed/unexposed subjects and polymorphism of different genes have been carried out. NORPPA (2004) and DHILLON *et al.* (2011) summarized published studies of genetic polymorphism impact on cytogenetic biomarkers in relation to different factors (environmental exposures, lifestyle and health status) as well impact on baseline level of markers.

In this study the relationship between GSTT1 and GSTM1 polymorphism and baseline MN frequency in PBLs of women from Serbia was investigated. Additionally, we studied the possible interaction between polymorphism and demographic/lifestyle factors in the modulation

of the baseline level of MN. To the best knowledge this is the first study of influence of polymorphism of these genes on the baseline level of cytogenetic biomarker in the population of the women from central Serbia.

In spite that in our study carriers of GSTT1 null and GSTM1 null genotype had higher MN frequency, the impact of these genes on genetic damages were not at a statistically significant level. Such results suggest that polymorphism of these genes might have no influence on the basal DNA damages in lymphocytes. The results of our study are in agreement with the results of several previous studies, who obtained no association between polymorphism in GSTT1 and GSTM1 genes and cytogenetic biomarkers in controls - MN and/or sister chromatid exchange (SCE) (TEIXEIRA *et al.*, 2004; WU *et al.*, 2000), chromosomal aberration (SCARPATO *et al.*, 1997), DNA tail moment (SINGH *et al.*, 2012, LIU *et al.*, 2006). Interestingly, recent pooled analysis revealed protective effect of GSTT1 null genotype in the total population (KIRSCH-VOLDERS *et al.*, 2006) Analyzing 90 healthy Japanese men, ISHIKAWA *et al.* (2004) did not observed influence of GSTT1 and GSTM1 polymorphism on the baseline frequency of MN. Apart in healthy individuals, neither of the GSTs genotypes (T1, M1 and P1) modulates the basal frequency of BMNM in thyroid cancer patients (HERNÁNDEZ *et al.*, 2006).

Overall, in present study individuals with dual null GSTT1/GSTM1 genotypes have significantly higher MN frequency in PBLs than other GSTs combinations. This result might indicate the impact of dual null combination on the genome integrity. It seems that these carriers might have reduced power of detoxification that finally leads to an increase of genetic damages. Similarly, SCARPATO *et al.* (1997) obtained that individuals with GSTT1 null and GSTM1 null genotype combination had the highest CA frequency, suggesting possible interaction between null genotypes. On the contrary, KIRSCH-VOLDERS *et al.* (2006) reported lower MN frequency in occupationally exposed individuals with both GSTT1 and GSTM1 genotypes than their positive counterparts.

The effect of lifestyle factors (e.g. smoking habits) on the genome integrity may be modified by the enzymes involved in detoxification. Tobacco smoke contains a multitude of genotoxic carcinogens (NORPPA, 2004). GSTM1 and GSTT1 gene encodes an enzymes involved in the metabolism of tobacco smoke constituents - polycyclic aromatic hydrocarbons (PAH), 1, 3-butadiene, ethylene oxide and halogenated alkanes (ALEXANDRIE *et al.*, 2004). NORPPA (2004) analyzing published studies indicated an increased susceptibility of the individuals with GSTM1 null genotype to genotoxic effect of tobacco smoke. Similarly, recently published research has revealed that a smokers lacking GSTM1 gene have statistically higher micronuclei compared to smokers with GSTM1 gene, while no association was found in nonsmokers (PALMA *et al.*, 2007). But still, SINGH *et al.* (2012) did not observed variation in DNA damages in nonsmokers, mild smokers and smokers with polymorphic GSTT1 and GSTM1 genotypes in both control subject and occupationally exposed workers. Among nonsmokers the level of CA was similar between null and positive individuals for both GSTT1 and GSTM1 genes, while smokers lacking the GSTM1 had statistically higher CA level (SCARPATO *et al.*, 1997). In particular, our results revealed significantly higher DNA damages in lymphocytes of smokers carrying GSTT1 null than their positive counterparts. Regarding GSTM1 genotype, GSTM1 null smokers as well as GSTM1 null nonsmokers have higher MN frequencies than positive counterparts, but the effect was not statistically significant. Generally, the presence of both null genotypes is associated with significantly higher MN frequency in smokers as compared to GSTT1positive/GSTM1positive

and GSTT1positive/GSTM1null, suggesting that these women have reduced detoxification capacity and that they are more sensitive to tobacco carcinogens.

Regarding the age, GSTT1 null genotype and GSTT1null/GSTM1null were associated with increased MN frequency in PBLs of women age >45 years. The protective effect of GSTT1 null genotype noticed in the study of KIRSCH-VOLDERS *et al.* (2006) was reversed in an age-dependent manner in total population as well in occupationally exposed group.

Considering the NDI values as marker of cell kinetics, we observed random effects of GST polymorphism.

In the present work, results suggest possible influence of dual null genotypes of GSTT1/GSTM1 on the basal MN frequency, as well influence on the level of MN in smokers and in women age >45 years. In addition, GSTT1 null genotype may have the potential to influence the baseline MN frequency in PBLs of smokers, as well as in women age >45 years. We believe that these results would allowed the understanding the role of genetic constitution on the expression of chromosomal damages, and that might be useful in explanation of interindividual variability in micronuclei frequency in peripheral blood lymphocytes.

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**UTICAJ POLIMORFIZMA GLUTATION – S TRANSFERAZE (GSTT1 I GSTM1) NA
BAZALNU FREKVENCU MIKRONUKLEUSA U LIMFOCITIMA PERIFERNE
KRVI**

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Izvod

U ovoj studiji analiziran je uticaj polimorfizma GSTT1 i GSTM1 gena na frekvencu mikronukleusa (MN) u limfocitima periferne krvi. Ukupno su bile uključene 134 žene iz Centralne Srbije. Polimorfizam GST gena je određivan primenom multipleks PCR-a, dok je citokinezis-blok mikronukleus (CBMN) metoda korišćena za utvrđivanje MN frekvence. Nosioci GSTT1 nultog i GSTM1 nultog genotipa imali su veću MN frekvencu u odnosu na nosioce pozitivnih genotipova, ali razlika nije bila statistički značajna. Osobe sa oba GSTT1/GSTM1 nulta genotipa imali su značajno veću frekvencu MN u odnosu na osobe sa kombinacijom pozitivan/pozitivan, pozitivan/nulti i nulti/pozitivan genotip. Pušači i žene >45 godina starosti sa GSTT1 nultim genotipom i GSTT1nultim/GSTM1nultim genotipom imale su statistički veću frekvencu MN u odnosu na osobe sa pozitivnim genotipovima. Rezultati studije ukazuju na mogući uticaj oba GSTT1/GSTM1 nulta genotipa na bazalnu MN frekvencu i na frekvencu MN kod pušača i žena starijih od 45 godina. Takođe, GSTT1 nulti genotip potencijalno utiče na bazalnu MN frekvencu u limfocitima periferne krvi pušača i žena >45 godina starosti.

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