INVESTIGATION OF THE ROLE OF NULL POLYMORPFISMS OF GSTM1 AND GSTT1 IN CUTANEOUS MALIGNANT MELANOMA

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ABSTRACT

Epidemiological investigations during the last decades have proven that the cutaneous malignant melanoma is a complex disease. There are variety of genetic and environmental factors and their interactions which are implicated in the developments of this neoplastic disease. The enhanced ultraviolet exposure (UV) is considered as the most important environmental factor contributing to skin melanoma. It has been fund that UV light has a pleiotropic effect on the skin cells, which includes both direct damage of DNA due to formation of pyrimidine (thymine) dimmers and indirect effect via generation of reactive oxygen species (ROS).

In this respect the gene variants in enzymes involved in detoxification of the oxidative stress products are considered as important factors influencing the risk for development of cutaneous malignant melanoma and other types of skin cancers promoted by UV exposure.

GSTT1 and GSTM1 are isoenzymes of the large family of glutathione-S-transferases, expressed in the skin and able to detoxify products of oxidative stress reactions caused by UV irradiation.

The aim of the current study was to examine the relation of GSTM1 and GSTT1 null polymorphisms with skin malignant melanoma risk in a pilot case-control study of Bulgarian patients and unaffected controls. A modified multiplex (duplex/triplex) PCR-based method was applied for detection of GSTs' genotypes.

Our results showed no statistically significant difference between melanoma patients and healthy controls in the frequency of null GSTM1 genotype (p=0.505, χ2-test). However there was a tendency for increased risk for melanoma in carriers of *GSTT1* null genotype (p=0.107, χ2-test), especially among the females who appeared to have more than five time higher risk than the women with non-null *GSTT1* genotype (OR=5.60, 95% CI, 1.54-20.43, p=0.014, Fisher's exact test).

Our current results suggest that the inherited absence of GST-theta, but not of GST-mu detoxifying enzymes due to the presence of homozygous null genotypes may be associated with skin malignant melanoma, especially in women.

Key words: *skin malignant melanoma, GSTM1, GSTT1, null polymorphism, genetic predisposition*

Introduction

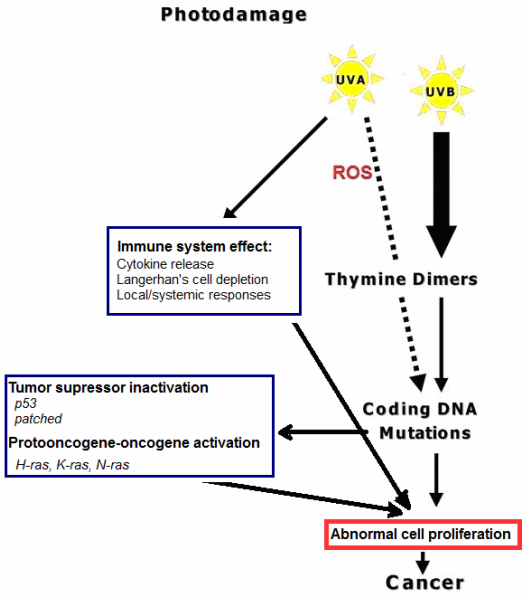
The skin malignant melanoma is a neoplastic disease which is developed during the tumor transformation f epidermal melanocytes. During the last two decades there is worldwide a stable tendency for significant increase of the cutaneous melanoma cases (Balch, Soong et al. 1983; Barth, Wanek et al. 1995; Brand, Ellwanger et al. 1997; Kamangar, Dores et al. 2006). Today, more than 350 new cases and more than 120 deaths of melanoma are registered each year in Bulgaria (Valerianova, Vukov et al. 2008).

Epidemiological investigations during the last decades have proven that the cutaneous malignant melanoma is a complex disease. There are variety of genetic and environmental factors and their interactions which are implicated in the developments of this neoplastic disease. The most important are considered those related to the behavior during exposure to sun light and the genetic determinants of the skin features and complexion (Elwood 1996; Gandini, Sera et al. 2005; Dennis, Vanbeek et al. 2008; Chang, Barrett et al. 2009). The enhanced ultraviolet exposure (UV) is

considered as the most important environmental factor contributing to skin melanoma, although the direct link between the risk and UV exposure is not a simple cumulative once: skin melanoma frequently is detected on skin rarely exposed to UV light or in persons which are not permanently exposed(Weinstock 1996; Gandini, Sera et al. 2005; Dennis, Vanbeek et al. 2008).

It has been fund that UV light has a pleiotropic effect on the skin cells, which includes both direct damage of DNA due to formation of pyrimidine (thymine) dimmers and indirect effect via generation of reactive oxygen species (ROS) (Figure 1, modified according to (Soehnge, Ouhtit et al. 1997; Kosmadaki and Gilchrest 2004)

Figure 1. Direct and indirect effect of UV light for carcinogenesis (modified according to (Soehnge, Ouhtit et al. 1997; Kosmadaki and Gilchrest 2004)



ROS can attack different types of target molecules such as lipids, proteins and nucleic acids. The carcinogenic effect of ROS is supposed to be due to the great amount DNA damages which can not be efficiently repaired by the repairing systems of the cells (Klaunig, Xu et al. 1998; Popov, Petkova et al. 2007 ; Klaunig, Kamendulis et al. 2009; Popov, Georgieva et al. 2011).

In this respect the gene variants in enzymes involved in detoxification of the oxidative stress products are considered as important factors influencing the risk for development of cutaneous malignant melanoma and other types of skin cancers promoted by UV exposure.

GSTT1 and GSTM1 are isoenzymes of the large family of glutathione-S-transferases, expressed in the skin and able to detoxify products of oxidative stress reactions caused by UV irradiation (Mossner, Anders et al. 2007).

GST-mu and GST-theta are two of the most relevant human GST isoenzymes, however they are genetically deleted in a high proportion of human population because of the homozygosity of non-fictional null alleles of their genes (GSTM1 and GSTT1), i.e. the homozygosity of the null alleles leads to complete lack of the enzyme activity of the respected isoenzyme (Cotton, Sharp et al. 2000; Hayes, Flanagan et al. 2005)..

GSTM1 together with the other four GSTM class members (GSTM, GSTM3, GSTM4 and GSTM5) are mapped to 1p13.3 (Pearson, Vorachek et al. 1993; McIlwain, Townsend et al. 2006; Laborde 2010). The close proximity of GSTM1 and GSTM2, as well as the presence of two almost identical 4.2-kb regions flanking the GSTM1 gene have been suggested to be the reasons for the observed entire GSTM1 gene deletion resulting in a null GSTM1 allele (GSTM1*0) (Pearson, Vorachek et al. 1993; Bolt and Thier 2006). The frequencies of GSTM1 alleles and genotypes display race and ethnic variations: 42% to 60% of Caucasians, 41% to 63% of Asians and only 16%

to 36% of Africans are homozygous for GSTM1*0 (null GSTM1 genotype) (O'Brien and Tew 1996; Cotton, Sharp et al. 2000; He, Connett et al. 2004; Hayes, Flanagan et al. 2005; Bolt and Thier 2006; McIlwain, Townsend et al. 2006; Gao, Cao et al. 2010).

Analogously to GSTM1, GSTT1 which is located at 22q11.2, consists of 5 exons and it is flanked by two highly homologous 18 kb regions (HA3 and HA5). The null GSTT1*0 allele is possibly caused by a homologous recombination resulting in 54 kb deletion containing the entire GSTT1 gene (Sprenger, Schlagenhaufer et al. 2000; Bolt and Thier 2006). A SNP (310A>C) in exon 3 of GSTT1 is the reason for substitution of Tre104 with Pro104 (Tre104Pro) in GST-theta protein, which was associated with a decrease in the catalytic activity possibly due to a conformational changes of the protein molecule (Alexandrie, Rannug et al. 2002). The frequency of the null GSTT1 genotype has also been found to vary significantly between different races and ethnic groups: between 13% and 31% (with some exceptions) in Caucasians in Europe and USA and between 35% and 48% in Asians (O'Brien and Tew 1996; Cotton, Sharp et al. 2000; He, Connett et al. 2004; Hayes, Flanagan et al. 2005; Bolt and Thier 2006; McIlwain, Townsend et al. 2006; Gao, Cao et al. 2010).

So far in the current literature there are only a limited number of studies reporting quite conflicting results for the effect of the null polymorphisms in GSTM1 and GSTT1 on the risk of malignant melanoma (Shanley, Chenevix-Trench et al. 1995; Kanetsky, Holmes et al. 2001; Dolzan, Rudolf et al. 2006; Mossner, Anders et al. 2007; Fortes, Mastroeni et al. 2011; Ibarrola-Villava, Martin-Gonzalez et al. 2012).

In this respect the aim of the current pilot study was to identify GSTM1 and GSTT1 genotype frequencies and to evaluate their impact on the susceptibility to cutaneous malignant melanoma in a Bulgarian population from Stara Zagora region.

Materials and methods

Patient and control populations

The patient group consisted of 24 patients with cutaneous malignant melanoma, who were enrolled in the Oncology center of Stara Zagora. Thirteen (54%) of the patients were males and the rest of 11 (46%) were females, all ranged between 42 to 77 years (median of 61 years).

The control group consisted of 124 healthy voluntaries or non-cancer hospital patients: 55 (44%) males and 69 (56%) females with an age ranging from 23 to 85 years (median of 60 years).

DNA exstraction

Genomic DNA of controls was isolated from cell mass of 2 ml of blood applying the conventional proteinase K digestion followed by protein precipitation with over-saturated solution of NaCl, and deposit of genomic DNA with absolute ethanol (Popov and Georgieva 2006). In some cases of controls and in all patients genomic DNA from 0.2 ml blood cell mass was isolated using a commercial kit for isolation of genomic DNA from blood (GenElute™ Mammalian Genomic DNA Miniprep Kit, Sigma, USA). The quality of the isolated DNA was evaluated by 1% agarose gel electrophoresis, and the quantity of the isolated DNA was measured spectrophotometrically.

Genotyping:

The null polymorphisms in *GSTM1* and *GSTT1* were assessed by a modified by us multiplex (duplex) PCR (Voso, D'Alo et al. 2002; Vlaykova, Gulubova et al. 2009). Some of the cases were genotyped by triplex PCR reaction (Figure 2). *GSTP1* was used as a referent gene for successful amplification and presence of sufficient amount of DNA template. In brief: two parallel PCRs for *GSTM1* and *GSTT1* polymorphisms with *GSTP1* as referent gene were carried out in a 25 µl mixture containing 2 to 4 µl of the genomic DNA, 1xPCR buffer, 2 mM $MgCl_2$ (for *GSTM1*) or 1.5 mM $MgCl_2$ (for *GSTT1*), 250 µM dNTP, 1U *Taq* polymerase (STS DNA polymerase, STS Ltd., Bulgaria) and 0.5 µM of the following primers: T1F (5'-TTC CTT ACT GGT CCT CAC ATC TC-3') and T1R (5'-TCA CCG GAT CAT GGC CAG CA-3') in the mix for *GSTT1*, M1F (5'-GAA CTC CCT GAA AAG CTA AAG C-3'), and M1R (5'-GTT GGG CTC AAA TAT ACG GTG G-

3') in the mix for *GSTM1* and P105F (5'- ACC CCA GGG CTC TAT GGG AA-3') and P105R (5'- TGA GGG CAC AAG AAG CCC CT-3') (Harries, Stubbins et al. 1997; Vlaykova, Miteva et al. 2007) for *GSTP1* in both mixtures. Amplification consisted of 10 min at 94^0C predenaturation and 40 cycles of denaturation at 94^0C for 1 minute, annealing at 62^0C for 30 seconds, and extension at 72^0C for 1 minute, followed by final extension at 72^0C for 7 minute . The triplex PCR was performed with primers for the three genes (GSTM1, GSTT1 and GSTP1). This results in a fragments of 459 bp for *GSTT1*, 219 bp for *GSTM1*, and 176 bp for *GSTP1* (Figure 2A and 2B). A positive and negative control, the latter containing water instead of DNA, was included in all PCRs. PCR products were analyzed on a 2.5% agarose gel, stained with ethidium bromide and documented with Gel documentation system (Syngene, Synoptics Ltd, UK).

Although this assay can not distinguish between heterozygote and homozygote positive genotypes, it conclusively identifies the homozygous null genotypes.

Statistical analyses:

Statistical analyses were performed using StatView v.4.53. for Windows (Abacus Concepts, Inc.). The frequencies of distribution in contingency tables were analyzed using $\chi 2$ test and Fisher's exact test. The differences in the non-normally distributed quantitative variables between unpaired groups were compared with Mann-Whitney U test. Factors with $p<0.05$ were considered statistically significant. The Odds ratio was calculated by using an interactive Online Software Package at the web site http://statpages.org/#Package (http://statpages.org/ctab2x2.html). If there were cells with a value of 0 in 2x2 contingency table, we applied the modification of Haldane for calculating of the OR (Miteva 2008):

OR=(2A+1)x (2D+1)/(2B+1)x(2C+1)

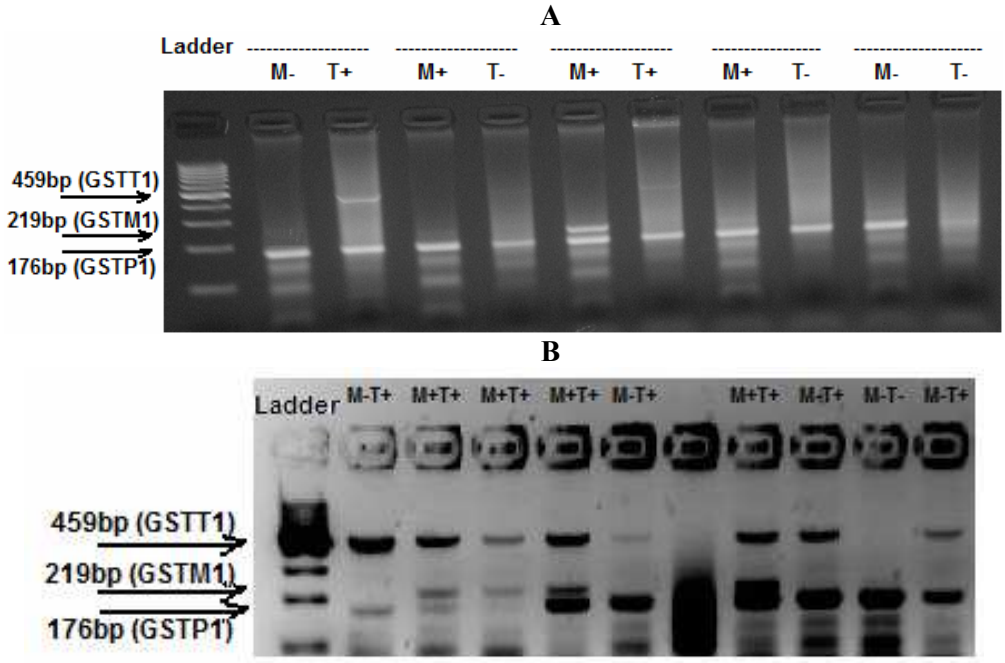
Results

For most of the cases the *GSTM1* and *GSTT1* null polymorphisms were analyzed in parallel duplex PCR reactions, using *GSTP1* as a referent gene in each of the reactions. Thus in the electrophoresis of the PCR reaction products of patients homozygous for *GSTM1* null polymorphism (*GSTM1 null*) there was visible only one band of 176 bp corresponding to the PCR product of the referent *GSTP1* gene, whereas the electrophoresis of the PCR products of DNA template from patients with one or two wide type alleles of *GSTM1* (*GSTM1 non-null*) presented two bands: 219 bp band of *GSTM1* PCR product and 176 bp band of *GSTP1* referent gene (Figure 2A).

Analogously, the electrophoretic profile of the PCR products from patients with at least one wide type *GSTT1* allele (*GSTT1* non-null) demonstrated two bans with a 459 bp band, corresponding to the *GSTT1* PCR product, and a band of 176 bp, corresponding to the *GSTP1* referent gene. The PCR products of genomic DNA from patients homozygous for the deleted *GSTT1* allele (GSTT1 null) were visible as a single 176 bp band of *GSTP1* referent gene only (Figure 2A).

The triple PCR reaction resulted in three bands of 459 bp, 219 bp and 176 bp if the person carries both GSTM1 and GSTT1 non-null genotypes; two bands of 459 bp and 176 bp if the person has GSTM null and GSTT1 non-null genotype; two bands of 219 bp and 176 bp if the person has GSTM non-null and GSTT1 null genotype, and one band of 176 bp if the person carries GSTM1 null and GSTT1 null genotypes (Figure 2B).

Figure 2. Genotyping for GSTM1 and GSTT1 null polymorphisms applying duplex (A) and triplex (B) PCR method.



Altogether in our preliminary study there were included a small cohort of 24 patients with cutaneous melanoma and a group of 124 controls consisted of voluntaries and non-cancer hospital patients.

The genotype frequencies of *GSTM1* and *GSTT1* null polymorphisms in patients with melanoma cases and controls are presented in Table 1 and Figure 3.

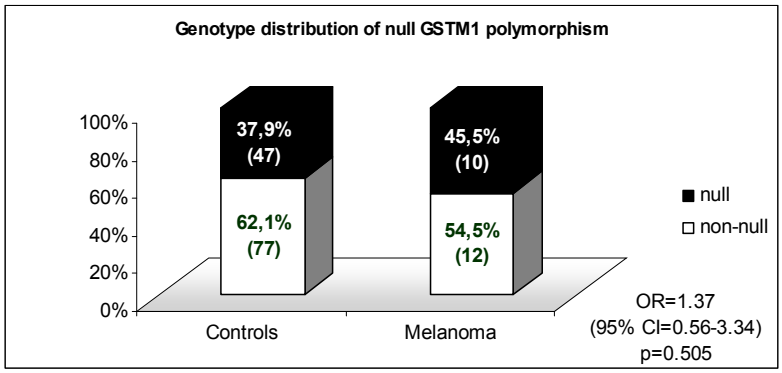
In the population studied of 124 non-cancer ethnic Bulgarian controls only 19 (15%) were homozygous for the null *GSTT1* allele and 47 (38%) for the null *GSTM1* allele. The distribution and the frequencies of the null genotypes among the melanoma patients were as the following: 7 (29%) for *GSTT1* null genotype and 10 (45%) for *GSTM1* null genotype (Table 1, Figure 3A and 3B). We did observed a statistically significant case-control difference in *GSTM1* homozygous null genotype frequencies (p=0.505, χ2-test) (Figure 3A) and only a tendency for prevalence of *GSTT1* null genotype in patients compared to the controls (p=0.107, χ2-test) (Figure 3B). Thus, compared with the referent non-null *GSTT1* genotypes, supposed to be the putative lowest risk group, the *GSTT1* null genotype tended to determined about 2-fold higher risk (OR=2.25, 95% CI, 0.85-6.05, p=0.107).

Table 1. Genotype frequencies of the *GSTM1* and *GSTT1* null genotypes in controls and patients with cutaneous malignant melanoma.

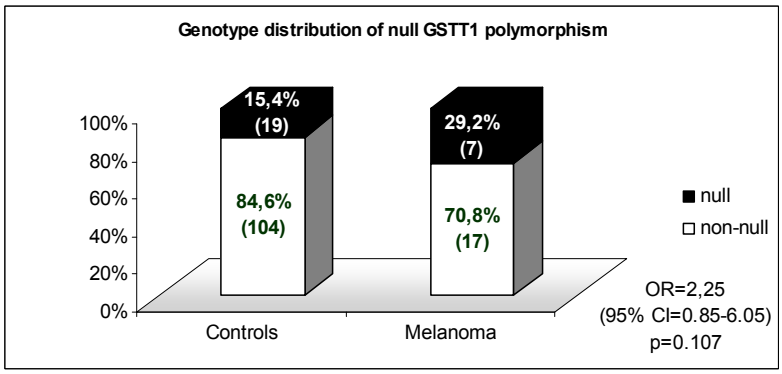
Genotypes	Patients		Controls		OR (95% CI), p-value
	n = 22/24	Frequency	n = 124/123	Frequency	
GSTM1 genotype frequency					
non-null	12	0.55	77	0.62	1.0 (referent)
null	10	0.45	47	0.38	1.37 (0.56-3.34), p=0.505
GSTT1 genotype frequency					
non-null	17	0.71	104	0.85	1.0 (referent)
null	7	0.29	19	0.15	2.25 (0.85 -6.05), p=0.107
Combined *GSTM1* and *GSTT1* genotype frequency					
non-null/non-null	21	0.875	118	0.95	1.0 (referent)
or null/non-null					
null/null (both null)	3	0.125	6	0.05	2.81 (0.72- 11.20), p=0.162

Figure 3. Genotype frequencies for GSTM1 (A) and GSTT1 (B) among melanoma patients and control individuals.

A

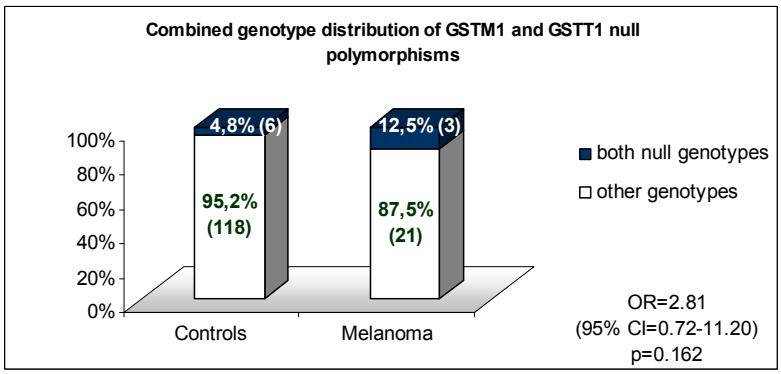


B



Considering that GST-mu and GST-theta isoenzymes have some overlapping substrate specificity, we analyzed the effect of the simultaneous lack of both enzymes due to carrying of double null genotypes (*GSTM1* null and *GSTT1* null genotypes). When the both null polymorphisms were analyzed in combination, we found that only 6 (5%) of controls had double null genotype (*GSTM1* null and *GSTT1* null), and 3 out of 24 (12.5%) melanoma patients were with such double null genotype (Figure 4). This could account for OR of 2.8 of the double null genotype obtained in the analysis (OR=2.8, 95% CI, 0.72- 11.20, p=0.162) (Table 1, Figure 4).

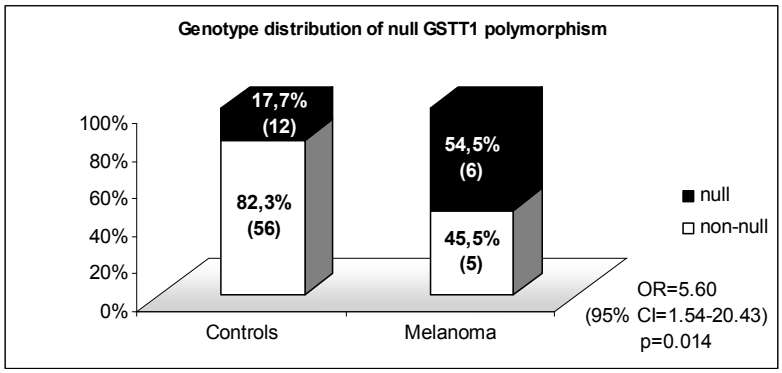
Figure 4. Combined genotype distribution of null GSTM1 and GSTT1 polymorphisms among melanoma patients and control individuals.



The presence of *GSTM1* and *GSTT1* null genotypes among the melanoma patients was analyzed in association with the gender. Interestingly we found that the null *GSTT1* genotype was more frequently observed in females (55%) than in males (8%, p=0.023, Fisher's exact test). No such difference in genotypes distribution among genders was seen in controls [18% (12 out of 68) *GSTM1* null genotype in females and 13% (7 out of 55) in males, p=0.453]. Based on the abovementioned observations, we found that the *GSTT1* null genotype accounted for significantly

higher more than 5-fold risk, for development of melanoma among women (OR=5.60, 95% CI, 1.54-20.43, p=0.014, Fisher's exact test). **(Figure 5).**

Figure 5. Frequencies of genotypes to *GSTT1* null allele in control and melanoma women.



Discussion

The development of cutaneous melanoma is a complex process and it is now widely accepted that risk for this aggressive and lethal disease is determined by an intricate interactions of both genetic and environmental factors such as susceptibility genes and UV light exposure (Kiyohara 2000). Inherited susceptibility is associated with high-penetrance genes such as *CDKN2A* (*p16I*^{NK4A}) and *Cdk4*, which are implicated in cell cycle regulation. Mutations in these genes have been detected in 20-30% of the relatives of the patients with familial malignant, which defines those mutations as pivotal predisposing factors for inherited familial malignant melanoma(Cannon-Albright, Kamb et al. 1996; Haluska and Hodi 1998; Holland, Schmid et al. 1999). However, those mutations are very rare in sporadic cutaneous melanoma, which accounts for about 90% of all cases (Bataille 2000; Bataille 2003).

That's why it as been suggested that several low-penetrance susceptibility genes may alter the predisposition to cutaneous melanoma. Studies for discovery of genetic factors responsible for development of sporadic melanoma are focused principally on genes and their protein products implicated in the regulation f skin tanning, DNA reparation, detoxification of oxidative metabolites and production of immune modulatory mediators (Ichii-Jones, Lear et al. 1998; Strange, Ellison et al. 1999; Lear, Smith et al. 2000; Kanetsky, Holmes et al. 2001; Wei, Lee et al. 2003; Landi, Kanetsky et al. 2005; Dolzan, Rudolf et al. 2006; Fargnoli, Argenziano et al. 2006; Mossner, Anders et al. 2007; Bishop, Demenais et al. 2009; Schoof, von Bonin et al. 2009)

Such low-penetrance susceptibility genes might be the genes encoding the detoxification of oxidative stress product, which are considered as important factors influencing the risk of cutaneous melanoma and other skin cancers associated with UV irradiation (Lear, Heagerty et al. 1996; Kerb, Brockmoller et al. 1997; Strange, Lear et al. 1998; Strange, Lear et al. 1998; Lear, Smith et al. 2000; Kanetsky, Holmes et al. 2001; Kerb, Brockmoller et al. 2002; Dolzan, Rudolf et al. 2006; Mossner, Anders et al. 2007; Povey, Darakhshan et al. 2007).

GSTM1 and *GSTT1* have been suggested as some of these low-penetrance susceptibility genes since they code for cytoplasmic GST-mu and GST-theta enzymes, which are involved in conjugation and in most cases detoxification with reduced glutathione of numerous electrophilic compounds with exogenic and endogenic origin including oxidative stress products (Hayes, Flanagan et al. 2005).

So far there are only limited number of studies exploring the role of GST family polymorphisms in melanoma, as the results are not entirely conclusive (Shanley, Chenevix-Trench et al. 1995; Kanetsky, Holmes et al. 2001; Dolzan, Rudolf et al. 2006; Mossner, Anders et al. 2007; Fortes, Mastroeni et al. 2011; Ibarrola-Villava, Martin-Gonzalez et al. 2012).

In the present work, we report our preliminary results from a relatively small case-control study on the impact of the null genotypes of *GSTM1* and *GSTT1* null polymorphisms on skin

malignant melanoma risk in Bulgarian population. Our preliminary study shows no significant effect of the null polymorphisms of *GSTM1* on the susceptibility to this neoplastic disease. Our results are in line with some other studies describing similar lack of association of *GSTM1* null polymorphism with the risk of malignant melanoma when studied independently (Shanley, Chenevix-Trench et al. 1995; Kanetsky, Holmes et al. 2001; Dolzan, Rudolf et al. 2006; Mossner, Anders et al. 2007; Fortes, Mastroeni et al. 2011; Ibarrola-Villava, Martin-Gonzalez et al. 2012). Similar negative results were reported also for the null GSTT1 genotype and for the combined carrying of both GSTM1 and GSTT1 null genotypes (Kanetsky, Holmes et al. 2001; Dolzan, Rudolf et al. 2006; Mossner, Anders et al. 2007; Fortes, Mastroeni et al. 2011; Ibarrola-Villava, Martin-Gonzalez et al. 2012). However in one of these studies the null *GSTM1* genotype, as well as the simultaneous GSTM1 and GSTT1 null genotypes, were more frequently found in melanoma patients with read and blond hair than the controls with the same hair type determining 2.2-forld (for GSTM1) and 9.5-fold (for GSTM1+GSTT1 null genotypes) higher risk for melanoma (Kanetsky, Holmes et al. 2001). Later it was reported by Fortes et al. that the risk of melanoma among the subset of participants who had both null GSTM1 and GSTT1 variants and experienced sunburns in childhood was extremely high, more that 9-fold (Fortes, Mastroeni et al. 2011).

In our preliminary study there was a tendency for increased risk for melanoma in carriers of *GSTT1* null genotype, especially among the females who appeared to have more than five time higher risk than the women with non-null *GSTT1* genotypes. We suppose that observed associations may be due to the enhanced UV-induced cutaneous damage seen in individuals harboring the null *GSTT1* genotype (Kerb, Brockmoller et al. 1997; Kerb, Brockmoller et al. 2002). However, a notable enlargement of the study populations will be required to guarantee the observed association of the risk for skin malignant melanoma with the *GSTT1* null genotype.

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