Genetic susceptibility to environmental carcinogenesis in Slovenian melanoma patients

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SUMMARY

The skin acts as the first defence barrier against external environmental pollutants, including chemicals and UV radiation. Cytochrome P450 CYP1A1 and glutathione S-transferases (GSTs) found in melanocytes and skin basal layers were shown to participate both in the metabolism of xenobiotics and in detoxification of reactive oxygen species (ROS). In our study we analysed the distribution of single and combined *CYP1A1*, *GSTM1*, *GSTT1* and *GSTP1* genotypes contributing to inter-individual differences in metabolism of xenobiotics and ROS in 125 Slovenian healthy individuals and in 140 patients with sporadic malignant melanoma.

Our results showed no statistically significant differences between melanoma patients and healthy controls in the frequency of polymorphic *CYP1A1* and *GST* genotypes. The risk of developing melanoma was not significantly increased in individuals homo- or heterozygous for the *CYP1A1*2A* allele combined with *GSTM1*0* genotype (OR: 1.86; 95 % CI: 0.36-7.71), but increased slightly in carriers of *CYP1A1*2A* combined with both *GSTM1*0* and *GSTT1*0* genotypes (OR: 3.42; 95 % CI: 0.36-29.6). Our results indicate that factors other than the polymorphic genes coding xenobiotic metabolising enzymes play a major role in protection against environmental carcinogenesis in human skin.

KEY WORDS

melanoma, cytochrome P450, glutathione S-transferase, detoxification, carcinogenesis

Introduction

It is generally accepted that UV exposure is the principal risk factor in the development of epithelial skin tumors, but its role in malignant melanoma is less well documented. Additionally other environmental and genetic factors may modify this risk (1). Mutations in DNA repair systems are known to result in skin-cancer prone syndromes such as Xeroderma pigmentosum and Cockayne syndrome. It has been suggested that a number of other genes may determine susceptibility to the development of malignant melanoma (2, 3), but the molecular basis of genetic events leading to melanoma is not yet clear.

Evidence of chemical carcinogenesis in melanoma is relatively slight, but epidemiological studies have indicated that workers in chemical industries are at an in-

creased risk of melanoma (4). In experimental animals, topically applied polycyclic aromatic hydrocarbons (PAHs) such as 7,12-dimethylbenz(a)anthracen (DMBA) have been strongly associated with increased risk for melanotic tumors (5). Cultured human melanocytes were found to be capable of metabolizing PAHs to more carcinogenic forms. Furthermore it was shown that PAHs and their metabolites bind to melanin and may in this way accumulate in melanocytes. Taking into account the bioactivating capacity of melanocytes it has been suggested that PAHs may play a role in the induction of melanoma (6).

In general, chemical carcinogens such as PAHs need to be metabolically activated in order to initiate mutagenesis and carcinogenesis. A large number of enzymes, most of which are polymorphic, participate in the metabolism of xenobiotics such as drugs and carcinogens (drug metabolising enzymes- DMEs) (7). Phase I DMEs, mostly cytochromes P450 (CYPs), metabolically activate precarcinogens to reactive electrophilic forms which can form DNA adducts if they have not been detoxified by Phase II DMEs such as glutathione S-transferases (GSTs). Genetic polymorphism of many enzymes involved in this process leads to inter-individual variations in the metabolism of precarcinogenic substances and could therefore represent a risk factor in cancer susceptibility (7). In particular, the enhanced activity of Phase I enzymes or the decreased activity of Phase II enzymes may result in higher amounts of activated carcinogen and the increased formation of DNA adducts (8, 9).

Aryl hydrocarbon hydroxylase (AHH) coded by the *CYP1A1* gene is the major Phase I DME involved in the metabolism of PAHs, which are not only present in tobacco smoke but also ubiquitous environmental pollutants. *CYP1A1* is expressed in the lungs and in peripheral lymphocytes as well as in the skin. Its expression in the skin is highly inducible by PAHs (10, 11). Solar radiation has also been shown to influence detoxification pathways in the skin (12), and UV exposure may also induce *CYP1A1* expression (13). A seasonal variation of CYP1A1 activity has been observed in human lymphocytes (14) and 10-fold higher induced AHH activities have been measured during the late summer and early fall as compared with activities six months later (15).

Genetic polymorphisms have been described for *CYP1A1*. The most common are substitutions in exon 7: Ile462Val (m2) and Thr461Asn (m4) and polymorphisms in the 3'-flanking region: T3801C (m1) and T3205C (m3), the latter being present only in African blacks. Conflicting data exist on the relationship of m1 and m2 polymorphisms with both catalytic activity (16, 17) and the ability to induce AHH (18, 19). Epidemiological studies suggested differences in the role of *CYP1A1* polymorphism in cancer susceptibility among ethnic groups (20–22). Individuals homozygous for m1 and m2 polymorphism have been reported to be at higher risk of lung cancer mostly in Japanese in which the frequencies of these mutations are much higher (16, 23).

Besides the enhanced activation of carcinogens by cytochrome P450 enzymes, the lack of detoxification by GSTs has also been linked with risk of cancer. Besides electrophilic metabolites of CYP1A1, GSTs also detoxify products of reactive oxygen species that are generated by UV irradiation and cause peroxidation of cellular proteins, lipids and DNA (24). GSTs and glutathione-peroxidases have been shown to be involved in the detoxification of reactive species produced during melanin synthesis. It has been proposed that selective inhibition of expression of GSTs in melanoma cell lines diminishes their capacity to scavange the reactive metabolites produced during melanin synthesis (25).

There are eight major classes of mammalian GSTs, which allow for a broad and overlapping substrate specificity. At least five of the human *GST* genes have been shown to be polymorphic. These polymorphisms are either gene deletions (*GSTM1* and *GSTT1*), or single nucleotide polymorphisms in the coding regions (*GSTM3*, *GSTP1* and *GSTZ1*) or in the promoter region (*GSTA1*) (26). Approximately 50% of the Caucasian population has inherited homozygous deficiency of *GSTM1* gene (*GSTM1*0/*0*) resulting in absent GST μ activity (27). The homozygous *GSTM1*0/*0* (*GSTM1* null genotype) has been associated with the high inducibility of *CYP1A1* gene transcription in human lymphoblasts (28).

GST μ has been found in the melanocytes of skin basal layers (29) and increased susceptibility for multiple basal cell carcinoma and sporadic melanoma seems to exist in individuals with a congenital deficiency of this isoenzyme (30). Besides *GSTM1,* the *GSTT1* null genotype has also been associated with increased susceptibility to cutaneous basal cell carcinoma (31, 32). Homozygous deficiency of *GSTM1* and *GSTT1* have also been associated with increased UV sensitivity of skin and with a more intense inflammatory reaction after UV irradiation (33).

The major GST isoenzyme expressed in melanocytes of the normal skin basal layer as well as in malignant melanoma belongs however to the π class GSTs and is coded by the *GSTP1* gene (29,34-36). Polymorphisms in exon 5 (Ile105Val) and exon 6 (Ala114Val) of the *GSTP1* gene have been reported as resulting in reduced conjugating activity (36). Individuals with the *GSTP1* 105Val allele have been reported to be at increased risk of breast and lung cancer by some (37, 38), but not by other studies (39,40). Increased skin tumorigenesis has been induced with DMBA in mice lacking the π class glutathione S-transferases (41). It is not known whether *GSTP1* polymorphism can modify the risk for melanoma in humans.

In the present study we analysed the distribution of single and combined genotypes of xenobiotic metabolising enzymes (*CYP1A1*, *GSTM1*, *GSTT1* and *GSTP1*) in healthy controls among the Slovenian population and in patients with sporadic malignant melanoma. We have also investigated whether the genetically determined imbalance between Phase I (CYP1A1) and Phase II (GSTs)

DMEs represents a risk factor for sporadic malignant melanoma in the Slovenian population.

Subjects and methods

Subjects

DNA samples from from 125 healthy individuals and 140 patients with sporadic malignant melanoma of which most had already been analysed for *CYP2D6* gene polymorphism in the Slovenian population (42) were included in the study. DNA samples from healthy subjects (73 males and 52 females) were obtained from the Blood Transfusion Center of Slovenia. The reference group of unrelated healthy controls was sampled for population immunogenetic studies (43) and was not selected according to age, gender or smoking habits since genotype distribution is not altered by such factors. Blood samples from patients with sporadic malignant melanoma (62 males and 78 females) were obtained at the Institute of Oncology, Ljubljana, Slovenia. The mean age in the patient group was 54 $\pm$ 13 (mean $\pm$ SD) years at the time of enrolment in the study. The clinical data considering the characteristics of the patients, and the location and the stage of the tumor were obtained from their medical records. A history of smoking was available only for half of the patients. The study design was approved by the Slovenian Ethics Committee for Research in Medicine.

Genotyping approach

The *CYP1A1* m1 polymorphism was initially analysed by PCR amplification following restriction with *Msp*I (16). The m2 and m4 polymorphisms were determined from the same PCR product digested either with *Bsr*DI or *Bsa*I respectively (20). The m3 polymorphism was analysed by PCR amplification of an 899-bp fragment followed by restriction with *Msp*I (20).

The deletions of *GSTM1* and *GSTT1* genes were detected using multiplex PCR. Both genes were simultaneously amplified in a single-step PCR reaction together with the β-globin gene as internal positive control (44). When a null allele was detected, only the gene in question and the β-globin gene were reamplified to reduce the chance of detecting null alleles due to preferential amplification of a single fragment.

Exon 5 and exon 6 of *GSTP1* gene were separately amplified from genomic DNA (36). Restriction with *Bsm*AI and *Cac*8I were used to identify Ile105Val and Ala114Val substitutions, respectively. Genotyping was randomly repeated in 10% of samples to check for the typing reliability.

Chi-square analysis using 3x2 and 2x2 contingency tables was used for frequency analysis of individual and combined *CYP1A1* and *GST* genotypes in healthy controls and melanoma patients. The odds ratio (OR) and the 95 % confidence interval (CI) were used to describe the strength of association of metabolic gene polymorphism with the risk of malignant melanoma (45).

Results

We used a PCR-based genotyping approach to investigate the possible genetic susceptibility to environmental carcinogenesis in malignant melanoma. We determined the frequency distribution of the polymorphic cytochrome P450- dependent monooxygenase *CYP1A1* and three glutathione S-transferases (*GSTM1*, *GSTT1* and *GSTP1*) genotypes in DNA samples from non-related healthy individuals and patients with sporadic malignant melanoma from Slovenian population.

The frequencies of *CYP1A1* alleles and *CYP1A1* genotypes in Slovenian healthy controls and melanoma patients are shown in Table 1. *CYP1A1* alleles were defined according to the recommended nomenclature (7, http://www.imm.ki.se/CYPalleles/). Polymorphic *CYP1A1* alleles were rare in Slovenian healthy population with frequencies of 0.048 for *CYP1A1*2A*, 0.048 for *CYP1A1*2B* and 0.040 for *CYP1A1*4* allele, respectively. The *CYP1A1*3* allele was not detected among the analysed healthy controls from the Slovenian population and for this reason we made no further investigation of it in the melanoma patients. The frequency of *CYP1A1*1/*2A* genotype was slightly, but not significantly higher in melanoma patients as compared to the healthy controls (OR: 1.46; 95% CI: 0.61-3.54); this was mostly due to the higher frequency of *CYP1A1*2A* allele in female melanoma patients compared to healthy female controls (OR: 2.10; 95% CI: 0.66-6.73). No statistically significant differences in the frequencies of either *CYP1A1* alleles or *CYP1A1* genotypes were observed between healthy controls and melanoma patients.

The frequencies of the *GSTM1* and *GSTT1* genotypes in melanoma patients and healthy controls are shown in Table 2. The genotyping method used did not allow us to detect heterozygous carriers of *GSTM1* or *GSTT1* gene deletion. The observed frequencies of homozygous *GSTM1* and *GSTT1* null genotypes in healthy controls (54.3 % and 24.1 %, respectively) were similar to those reported for other Caucasian populations (44).

The *GSTM1* null genotype was present in 52.6% of melanoma cases indicating that carriers of the *GSTM1* null genotype were not at increased risk of melanoma (OR: 0.932; 95% CI: 0.57-1.53). No differences were observed in the frequency distribution of the *GSTM1* null genotype between men and women, either in melanoma patients or in healthy controls (data not shown). A slight but not significant under-representation of *GSTT1* null genotype was observed in melanoma patients as compared to the controls (OR: 0.736; 95% CI: 0.40-1.34). When stratified by sex, the frequency of the *GSTT1* null genotype was also slightly, but not significantly, lower in both healthy women (OR: 0.698; 95% CI: 0.29-1.68) and in female melanoma patients (OR: 0.708; 95% CI: 0.301-1.66) when compared with the respective male groups. Also the frequencies of combined *GSTM1* and *GSTT1* null genotypes were slightly, although not significantly, lower in melanoma patients as compared to the healthy controls (OR: 0.512; 95% CI: 0.21-1.23).

Table 1. Frequencies of polymorphic cytochrome P450 *1A1 (CYP1A1)* alleles and genotypes in melanoma patients and healthy controls.

Nucleotide position					Melanoma		Healthy controls	
CYP1A1	**2453**	**2455**	**3205**	**3801**	**n=140**		**n=125**	
Allele	(m4)	(m2)	(m3)	(m1)	n	frequency	n	frequency
**1*	C	A	T	T	239	0.8535	216	0.864
**2A*	C	A	T	C	17	0.061	12	0.048
**2B*	C	G	T	C	9	0.032	12	0.048
**3*	C	A	C	T	-	-	0	0
**4*	A	A	T	T	7	0.0535	10	0.040
***CYP1A1* genotype**								
**1/*1*					101	0.722	95	0.760
**1/*2A*					14	0.100	9	0.072
**1/*2B*					9	0.064	10	0.080
**2A/*2A*					1	0.007	1	0.008
**2A/*2B*					0	0	0	0
**1/*4*					14	0.100	7	0.056
**2A/*4*					1	0.007	1	0.008
**2B/*4*					0	0	2	0.016

The distribution of polymorphic *GSTP1* genotypes is shown in Table 3. Also the frequencies of *GSTP1* genotypes in Slovenian population were found to be similar to those reported for other Caucasian populations (36, 46). The frequencies of *GSTP1* genotypes in melanoma patients were not significantly different from those observed in healthy controls, however the frequency of the *GSTP1*B/*B* genotype was slightly over-represented in melanoma patients (OR: 2.44; 95 %CI: 0.62-9.68). This was mostly due to the increased *GSTP1*B/*B* genotype in female patients as compared to female healthy controls (OR: 2.57; 95 %CI: 0.48-13.7). As both *GSTP1*B* and **C* alleles were reported to have decreased conjugating capacity (34), *GSTP1* genotypes were grouped for further analysis according to the presumed enzymatic activity as shown in Table 3. No significant difference in presumed enzymatic activity was observed between melanoma patients and healthy controls, indicating that individuals with low GST π conjugating activity are not at increased risk of melanoma (OR: 1.4; 95 % CI: 0.64-3.06).

We also investigated if imbalance between Phase I and Phase II detoxification increases the susceptibility for melanoma. The risk to develop melanoma was not significantly increased in individuals homo- or heterozygous for *CYP1A1*2A* allele combined with *GSTM1* null genotype (OR: 1.86; 95 % CI: 0.36-7.71), but increased slightly in carriers of *CYP1A1*2A* combined with both *GSTM1* and *GSTT1* null genotypes (OR: 3.42; 95 % CI: 0.36-29.6). It was not possible to asses the effect of combined *CYP1A1*2A* allele with the *GSTP1* low activity genotypes as only one individual in each, patients and control, group carried this combination.

Table 2. Frequencies of glutathione S-transferase M1 and T1 (*GSTM1* and *GSTT1*) genotypes in melanoma patients and healthy controls.

***GST* genotype**	**Melanoma (n = 137)**		**Healthy controls (n = 116)**	
	n	frequency	n	frequency
GSTM1 present	65	0.474	53	0.457
GSTM1 null	72	0.526	63	0.543
GSTT1 present	111	0.819	88	0.759
GSTT1 null	26	0.190	28	0.241
Combined *GSTM1* and *GSTT1* genotypes				
Both present	48	0.350 (48)	39	0.336
Either present	80	0.584 (80)	63	0.543
Both null	9	0.066 (9)	14	0.121

Table 3. Distribution of polymorphic glutathione S-transferase P1 *(GSTP1)* genotypes.

***GSTP1* genotype**			**Melanoma (n = 122)**		**Controls (n = 110)**	
	codon 105	**codon 114**	**n**	**frequency**	**n**	**frequency**
**A/*A*	Ile	Ala	59	0.484	54	0.491
**B/*B*	Val	Ala	8	0.066	3	0.027
**C/*C*	Val	Val	0	0	4	0.036
**A/*B*	Ile/Val	Ala	24	0.197	27	0.245
**B/*C*	Val	Ala/Val	9	0.074	5	0.046
**A/*C*	Ile/Val	Ala/Val	22	0.180	17	0.155
presumed activity	***GSTP1* genotype**					
high	**A/*A*		59	0.484	54	0.491
high/low	**A/*B, *A/*C*		46	0.377	44	0.400
low	**B/*B, *C/*C, *B/*C*		17	0.139	12	0.109

Discussion

The skin acts as the first defence barrier against external environmental pollutants, including chemicals and solar UV radiation. Both CYP1A1 and GSTs were found in melanocytes and epidermal basal layers (10,11, 29,34,35) and were shown to participate both in the metabolism of xenobiotics such as PAHs and in the detoxification of reactive species (24,25). Although the UV irradiation that generates the reactive oxygen species and causes peroxidation of cellular proteins, lipids and DNA appears to be one of the major causes of epithelial skin tumors (12), previous studies have indicated that exposure to chemical carcinogens may also represent a risk factor for melanoma (4-6). Malignant melanoma of the skin is the tenth most common cancer in the Slovenian population. Its incidence has almost doubled in the last ten years and has reached the incidence of 11.9 and 13.2 per 100.000 men and women, respectively (47).

In our study we analysed the genotype distribution of polymorphic *CYP1A1* and *GSTs* that contribute to inter-individual differences in the metabolism of carcinogens to evaluate whether susceptibility to environmental carcinogenesis modifies the risk of melanoma in the Slovenian population. The frequency of *CYP1A1* alleles as well as *CYP1A1* genotypes in the Slovenian healthy population were similar to those reported for other Caucasian populations (20,46). Although the frequencies of *CYP1A1*2A* and *GSTP1*B/*B* genotypes were slightly over-represented in melanoma patients, our results show no statistically significant differences in the frequencies of polymorphic genotypes between melanoma patients and healthy controls. In contrast with the published biochemical data indicating higher GST π activity and enzyme content in female skin as compared to male skin (34), we found no significant differences in the distribution of polymorphic *GSTP1* alleles in the leukocytes of males and females among either healthy controls or melanoma patients. The risk of developing melanoma was the highest in carriers of *CYP1A1*2A* combined with both *GSTM1* and *GSTT1* null genotypes. Although the risk of developing melanoma depends on many genetic factors it may increase with the number of susceptibility alleles.

The large amount of published data suggests that susceptibility to epithelial skin tumors is dependant not only on UV exposure, but also on host genetic factors. The role of genetic factors is most clearly evident from observations of familial predisposition in about 10% of melanoma cases. The cyclin-dependent kinase inhibitor-2A gene (*CDKN2A*) is one of the major susceptibility genes within familial malignant melanoma and a novel mutation of this gene was recently described in Slovenian melanoma kindred (48). Genetic factors also determine the capacity for xenobiotic metabolism (49, 50). Most of the positive associations between genetically determined xenobiotic metabolising capacity and basal cell carcinoma were observed after stratifying patients according to the number of tumors (31,32) and tumor site (51,52). Some data indicates that among persons with hair colour traditionally associated with increased risk for melanoma absence of both *GSTM1* and *GSTT1* further elevates melanoma risk (53). Also the analysis of the GSTM1 phenotype in the Spanish population indicated a 2-fold higher risk of developing malignant melanoma in individuals lacking GSTM1 activity in peripheral blood lymphocytes (30). On the other hand, a large number of studies using a genotyping approach suggested that *GSTM1* null or *GSTT1* null genotypes do not relate to increased susceptibility for melanoma (54,55), familial basal cell carcinoma (55) or solar keratoses (56). Although it has also been reported that genetic polymorphism of other Phase I detoxifying enzymes like CYP2D6 are associated with increased susceptibility to melanoma (57,58), our previous study revealed no statistically significant difference in the distribution of CYP2D6 poor metabolisers between Slovenian melanoma patients and healthy controls (42).

One possible limitation of our study was that the healthy control group represented the genotype distri-

bution in the general population and was not matched to melanoma patients according to age and sex. Although not enough information is available whether these parameters modify melanoma risk, it is not likely that they would influence genotype distribution. As the smoking status was available only for half of the melanoma patients (5 smokers, 59 non-smokers) we were not able to investigate any correlation between exposure to cigarette smoking and the risk of melanoma in relation to the metabolic capacity of the carcinogens present in tobacco smoke, but epidemiological studies have not found that the risk of melanoma is significantly influenced by smoking tobacco (59). The results obtained in our present study may have been influenced by the limited number of patients available, which did not allow for the stratification of patients according to tumor location, number or stage, and for this reason they may be considered preliminary. This limitation regarding the small number of patients might be overcome in the future as the original data relating to most of the melanoma patients and healthy controls have been submitted to the database of the International collaborative study on genetic susceptibility to environmental carcinogenesis (GSEC) to allow for the pooled analysis of a large number of patients in the future (60).

In conclusion, the results of our study indicate that in the Slovenian population polymorphic genes coding for xenobiotic metabolising enzymes does not play a major role in protection against environmental carcinogenesis in human skin. Despite the low frequencies of polymorphic alleles of *CYP1A1* in the Slovenian population these genetic polymorphisms may result in an increased susceptibility to melanoma in individuals with higher exposure to PAHs, either because of their professional exposure or due to topical treatment with ointments containing tar in some skin disorders.

Acknowledgements

The authors wish to thank Blanka Vidan-Jeras from the Tissue Typing Center, and the Blood Transfusion Center of Slovenia for providing DNA samples from healthy controls. This work was financially supported by The Slovenian Research Agency (ARRS Grant No. PO-0503-0381).

Abbreviations

AHH - aryl hydrocarbon hydroxylase
CDKN2A-cyclin-dependent kinase inhibitor-2A
CI- confidence interval
CYP- cytochrome P450
DME- drug metabolizing enzyme
GST- glutathione S-transferase
OR- odds ratio
PAHs- polycyclic aromatic hydrocarbons
ROS- reactive oxygen species

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