ORIGINAL ARTICLE

Droplet Digital PCR Analysis of *GSTM1* Deletion Polymorphism in Psoriatic Subjects Treated with Goeckerman Therapy

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Summary: Goeckerman therapy (GT) represents an effective treatment of psoriasis including a combination of pharmaceutical grade crude coal tar (CCT) and ultraviolet irradiation (UV-R). Coal tar contains a mixture of polycyclic aromatic hydrocarbons. The best known carcinogenic polyaromate – benzo[a]pyrene is metabolized into a highly reactive benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE). Glutathione S-transferase M1 (GSTM1) catalyses the conjugation of drugs, toxins and products of oxidative stress with glutathione. The aim of the study is to found possible associations between *GSTM1* genotypes and the level of BPDE-DNA adducts in 46 psoriatic patients treated with GT. For genotyping, droplet digital PCR was applied. The *GSTM1* copy number was normalized to β-globin reference gene. In five *GSTM1*1/*1* subjects, the *GSTM1* to β-globin ratio moved from 0.99 to 1.03 with a median of 1.01. *GSTM1*0/*1* heterozygotes (n = 20) contained only one *GSTM1* function allele which conditioned the ratio 0.47–0.53 (median 0.50). *GSTM1*0/*0* individuals (n = 21) showed no amplification of the null variants because of the large deletion in *GSTM1*. BPDE-DNA concentrations ranged from 1.8 to 66.3 ng/µg with a median of 12.3 ng/µg. *GSTM1*0/*0* and *GSTM1*0/*1* genotypes showed non-significantly higher concentrations of BPDE-DNA adducts than the *GSTM1*1/*1* one (12.3 and 12.4 vs 7.8 ng/µg). The non-significant relationship between BPDE-DNA adducts and *GSTM1* genotypes in psoriatic patients could be associated with relatively low doses of CCT and short-term UV-R exposures used in GT.

Keywords: *GSTM1; Psoriasis; Goeckerman therapy; Genotyping; BPDE-DNA adducts*

Introduction

Goeckerman therapy (GT) represents an effective treatment of psoriasis including a combination of pharmaceutical grade crude coal tar (CCT) ointment and ultraviolet irradiation (UV-R). This therapeutic approach is applied in cases of light to moderately severe forms of psoriasis (1). CCT contains a mixture of polycyclic aromatic hydrocarbons (PAHs). The best known carcinogenic polyaromate – benzo[a]pyrene (BaP) is metabolized into a highly reactive benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE) and other reactive species.

The conjugation of BaP derivatives is catalyzed by glutathione S-transferases GSTM1 or GSTP1, and UDP glucuronosyltransferases 1A10, 1A6, 1A7C or 1A9. However, BPDE also intercalates in DNA by forming covalent bond with the nucleophilic guanine nucleotide bases at the N2 position and creates the BPDE-DNA adduct (2). GSTM1 (EC 2.5.1.18) is a cytosolic enzyme which catalyses the conjugation of drugs, toxins and products of oxidative stress

http://dx.doi.org/10.14712/18059694.2016.94
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with glutathione to form less reactive and more easily excreted water-soluble metabolites. To ensure high effectiveness of the conjugations, the *GSTM1* gene (chromosome location 1p13.3) is expressed in a lot of human tissues including liver and skin (3).

Previously published papers showed that genetic polymorphisms in *GSTM1* conditioned the individual response to electrophilic xenobiotic substances including PAHs. A large deletion (*GSTM1*0* null variant) occurs hereditarily in about 50% of Caucasian population. The absence of active enzyme in subjects with the *GSTM1*0/*0* genotype declines the efficiency of detoxification processes and leads to genotoxicity, toxic encephalopathy, higher cutaneous ultraviolet radiation erythemal sensitivity and risk of asbestosis or cancer (4–10). It is apparent that the activity of GSTM1 influences the level of BPDE and hence also the level of BPDE-DNA adducts. However, the results of studies focused on relationships between the GSTM1 activity and the level of BPDE-DNA adducts, are still inconsistent (11–13). Moreover, only a little is known about genotoxic and mutagenic risks associated to *GSTM1*0/*1* heterozygosity (10).

Genetic analysis of the *GSTM1* deletion polymorphism is usually performed *via* Southern blotting, long range PCR, and real-time PCR with either SYBR Green or specific hydrolytic probes (14–17). All these methods distinguish the *GSTM1*0/*0* genotype from *GSTM1*1/*1* and *GSTM1*0/*1* ones. Quantitative real-time PCR enables separation of *GSTM1*1/*1* and *GSTM1*0/*1*; both these genotypes featured by specific copy number reveal different values of cycle threshold (*Ct*). To normalize the input amount of genomic DNA, the *Ct* values are related to a reference gene with the constant copy number in the genome: *albumin*, β-globin or *RNAse P* (10, 18, 19). Despite the use of sophisticated expectation-maximisation algorithms and precisely defined cut-off *Ct* intervals for *GSTM1*1/*1*, **0/*1* and **0/*0*, the results of trimodal genotyping are not clear and strictly depend on DNA quality and PCR amplification efficiency.

Droplet digital PCR (ddPCR) is a modern technology amplifying DNA separately in thousands of nanoliter-sized oil microdroplets. After PCR, the fluorescence of each droplet is recorded and the total number of events (droplets) above the threshold is counted. Here we describe a novel approach to *GSTM1* genotyping based on droplet digital PCR. Using this technique we investigated a cohort of psoriatic patients treated with GT. In them, elevated level of BPDE-DNA adducts were previously found (20).

Material and Methods

The cohort created a group of 46 patients with chronic stable plaque psoriasis treated with GT. The group consisted of 22 males and 24 females (average age of 48 years, age span 20–82 years; 23 smokers and 23 non-smokers). Within GT therapy, dermatological ointment containing 3% of CCT was administered daily overnight on psoriatic lesions. According to the extent of lesions, 18–62% of the total body surface was covered by CCT ointment. Each morning the residues of CCT were removed from the body (using oil bath) and the patients were whole-body irradiated by UV-R. The density of used radiation was 249.75 μW/cm^2 of UV-B and 131.8 μW/cm^2 of UV-A. The effectiveness of the therapy was calculated from basic characteristics of actual disease status (erythema, desquamation, and skin infiltration) and expressed as the PASI score (Psoriasis Area and Severity Index). The study was approved by the Ethics Committee of the Charles University Hospital in Hradec Králové, Czech Republic. Written informed consent was obtained from each patient.

EDTA-treated peripheral blood specimens were collected immediately after GT. Genomic DNA was extracted from 200 μL of blood with a QIAamp DNA Blood Mini Kit (Qiagen, Germany). The level of BPDE-DNA adducts was determined by using the standard method OxiSelect BPDE-DNA Adduct ELISA Kit (Cell Biolabs, USA). The results were expressed in nanograms of BPDE-DNA adducts per microgram of DNA.

For genotyping, droplet digital PCR (QX100 Droplet Digital PCR System, Bio-Rad, USA) was applied. The *GSTM1* copy number was normalized to β-globin reference gene. The amplification mix (25 μL) contained 12.5 μL 2× concentrated ddPCR Supermix (Bio-Rad, USA), 900 nM of each primer, 250 nM of hydrolysis fluorescent probes, and 100 ng of DNA. The sequences of primers and probes were as follows: *GSTM1* forward primer 5′-CAC CTG CAT TCG TTC ATG TGA C-3′, *GSTM1* reverse primer 5′-AAG CAA GAG CAG AGA GGA GAC-3′, *GSTM1* hydrolytic probe 5′-FAM-TTC AGT CCT GCC ATG AGC AGG CAC A-BHQ1-3′, β-globin forward primer 5′-GAG GGT TTG AAG TCC AAC TCC TAA-3′, β-globin reverse primer 5′- CAG GGT GAG GTC TAA GTG ATG ACA-3′, and β-globin hydrolytic probe 5′-HEX-CAG TGC CAG AAG AGC CAA GGA CAG GT-BHQ1-3′.

The data were statistically processed by the R software version 3.22 using the "*nortest*" and "*psych*" packages. Because the Anderson-Darling test for the normality had rejected the hypothesis of a normal distribution of the BPDE-DNA adducts, nonparametric one-side Wilcoxon tests was used. Differences were considered to be statistically significant when $P < 0.05$.

Results and Discussion

Droplet digital PCR enabled identification of all three genotypes. As illustrated in Fig. 1, there were no problems to evaluate the proper *GSTM1* genotype if normalization to β-globin gene was performed. In five *GSTM1*1/*1* subjects, the *GSTM1* to β-globin ratio moved from 0.99 to 1.03 with a median of 1.01 proving the presence of two function alleles of *GSTM1* in diploid cells. *GSTM1*0/*1* heterozygotes (n = 20) contained only one *GSTM1* function allele which conditioned the ratio 0.47–0.53 (median 0.50). *GSTM1*0/*0*

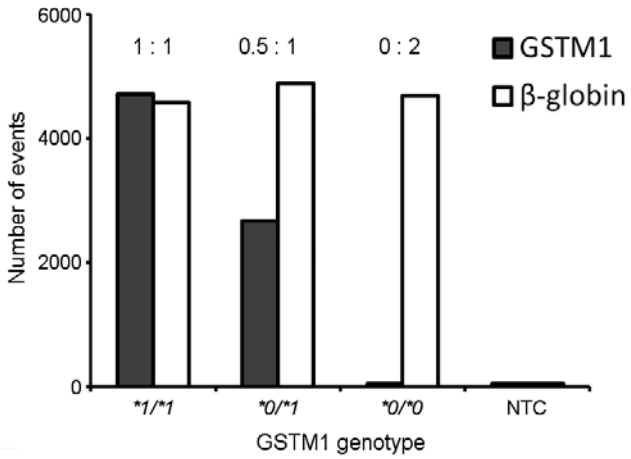

Fig. 1: The number of events recorded by ddPCR in different *GSTM1* genotypes (black) normalized to β-globin gene (white). The proper *GSTM1* to *β-globin* copy number ratio is indicated above the columns; NTC means no template control.

individuals (n = 21) showed no amplification of the null variants because of the large deletion in *GSTM1*.

The prevalence of *GSTM1*0* was 0.67, and the genotype frequencies were in agreement with the Hardy-Weinberg equilibrium. The frequency of *GSTM1*0* determined in the patients agreed with the results of other studies (10, 18).

BPDE-DNA adducts were detected in all investigated blood specimens. Their concentrations ranged from 1.8 to 66.3 ng/µg with a median of 12.3 ng/µg. No significant associations between the levels of adducts and sex or smoking were observed. *GSTM1*0/*0* and *GSTM1*0/*1* genotypes showed non-significantly higher concentrations of BPDE-DNA adducts in blood cells than the *GSTM1*1/*1* one (median values 12.3 and 12.4 vs 7.8 ng/µg, Table 1). Combinating *GSTM1*1/*1* and *GSTM1*0/*1* genotypes into one group, the difference in DNA adducts completely disappeared (median 12.2 ng/µg; $P = 0.261$). This fact could clearly justify the importance of trimodal genotyping *GSTM1*.

Our data show that the concentrations of BPDE-DNA adducts in blood cells of psoriatic subjects in GT do not significantly associate with the *GSTM1* genotype, though elevated levels of BPDE-DNA adducts in *GSTM1*0/*0* and *GSTM1*0/*1* carriers in comparison with the *GSTM1*1/*1* were apparent.

We assume that this non-significant relationship could be associated with relatively low doses of CCT and short-term UV-R exposures used in GT. In subjects exposed in industry to higher doses of PAHs, the relationship between BPDE-DNA adducts and *GSTM1* was more evident (11, 12, 21, 22). Further, in parallel with GSTM1, the BaP derivates may also be conjugated by UDP glucuronosyltransferases to corresponding glucuronides. Therefore, if we assess the association between the GSTM1 activity and the level of BPDE-DNA adducts, we have to take into account the fact that we evaluate only a part of genetically conditioned processes of activation and deactivation of BaP (23, 24). Finally, the skin of psoriatic patients was described to have lower GSTM1 catalytic activity than the normal skin (25). The loss of GSTM1 activity could lead to decreased expression of other GSTM isozyme (26) and to compensatory induction of other PAHs metabolizing enzymes, e.g., GSTP1 (27) or CYP1A1, especially in *GSTM1*0/*0* homozygotes (11, 28).

Tab. 1: DNA adducts in *GSTM1* genotypes.

***GSTM1* genotypes**	**n**	**BPDE-DNA (ng/µg)**		***P***
		Median	**Range**	
*GSTM1*0/*0*	21	12.3	1.9–66.3	*ref.*
*GSTM1*0/*1*	20	12.4	1.8–50.1	0.329
*GSTM1*1/*1*	5	7.8	7.5–49.7	0.227
*GSTM1*1/*1* or *GSTM1*0/*1*	25	12.2	1.8–50.1	0.261

ref. reference group

Conclusions

Droplet digital PCR has proved to be suitable for analysis of *GSTM1* deletion polymorphism. At the group of psoriatic patients treated with GT, we found non-significant differences in the levels of BPDE-DNA adducts, roughly corresponding to genetic polymorphisms in *GSTM1*. Taking into account all the factors mentioned above, a larger population study evaluating the associations between BPDE-DNA adducts and other xenobiotic metabolizing enzyme polymorphisms in psoriatic patients should be performed.

Acknowledgements

This work is supported by the projects PRVOUK P37/09 and PRVOUK P37/11 of Charles University, Faculty of Medicine in Hradec Králové, Czech Republic.

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Received: 31/05/2016
Accepted: 13/06/2016