Droplet Digital PCR Analysis of \textit{GSTM1} Deletion Polymorphism in Psoriatic Subjects Treated with Goeckerman Therapy

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\textbf{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 (BaP) 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 \textit{GSTM1} genotypes and the level of BPDE-DNA adducts in 46 psoriatic patients treated with GT. For genotyping, droplet digital PCR was applied. The \textit{GSTM1} copy number was normalized to \textit{β}-globin reference gene. In five \textit{GSTM1*1/*1} subjects, the \textit{GSTM1} to \textit{β}-globin ratio moved from 0.99 to 1.03 with a median of 1.01. \textit{GSTM1*0/*1} heterozygotes (\textit{n} = 20) contained only one \textit{GSTM1} function allele which conditioned the ratio 0.47–0.53 (median 0.50). \textit{GSTM1*0/*0} individuals (\textit{n} = 21) showed no amplification of the null variants because of the large deletion in \textit{GSTM1}. BPDE-DNA concentrations ranged from 1.8 to 66.3 ng/µg with a median of 12.3 ng/µg. \textit{GSTM1*0/*0} and \textit{GSTM1*0/*1} genotypes showed non-significantly higher concentrations of BPDE-DNA adducts than the \textit{GSTM1*1/*1} one (12.3 and 12.4 vs 7.8 ng/µg). The non-significant relationship between BPDE-DNA adducts and \textit{GSTM1} genotypes in psoriatic patients could be associated with relatively low doses of CCT and short-term UV-R exposures used in GT.

\textbf{Keywords:} \textit{GSTM1}; Psoriasis; Goeckerman therapy; Genotyping; BPDE-DNA adducts

\textbf{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 \textit{GSTM1} or \textit{GSTM1}, 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). \textit{GSTM1} (EC 2.5.1.18) is a cytosolic enzyme which catalyses the conjugation of drugs, toxins and products of oxidative stress.
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 electrophile 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 decreases 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/*0 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 trinodal 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/cm² of UV-B and 131.8 μW/cm² 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′-AAC CAA GAG CAG CAC-3′, GSTM1 hydrolytic probe 5′-FAM-TTC AGT CCT GCC ATG AGC AGG CAC A-BHQ1-3′, β-globin forward primer 5′-GAG CTT TTG AAG TCC AAC TCC TAA-3′, β-globin reverse primer 5′- CAG GGT GAG GTC TAA GTG ACA-3′, and β-globin hydrolytic probe 5′-HEX-CAG TGC CAG AAC AGG 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
adducts in blood cells of psoriatic subjects in GT do not to 66.3 ng/µg with a median of 12.3 ng/µg. No signifi-
mable blood specimens. Their concentrations ranged from 1.8 to 66.3 ng/µg with a median of 12.3 ng/µg. No signifi-
cant associations between the levels of adducts and sex or
smoking were observed. GSTM1*0/*0, 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).
Combining GSTM1*1/*1 and GSTM1*0/*1 genotypes into one group, the difference in DNA adducts complete-
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 deriva-
tes may also be conjugated by UDP glucuronosyltransfera-
tions 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 pro-
cesses 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).

Conclusions

Droplet digital PCR has proved to be suitable for anal-
ysis 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 popu-
lation study evaluating the associations between BPDE-DNA
adducts and other xenobiotic metabolizing enzyme polymor-
phisms in psoriatic patients should be performed.

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References

1. Moscaliuc ML, Heller MM, Lee ES, Koo J. Goeckerman therapy: a very effective,
yet often forgotten treatment for severe generalized psoriasis. J Dermatolog Treat
2013; 24: 34–37.
2. Borska L, Andrys C, Krejsek J, et al. Oxidative damage to nucleic acids and
benzo(a)pyrene-7,8-diol-9,10-epoxide-DNA adducts and chromosomal aberration
in children with psoriasis repeatedly exposed to crude coal tar ointment and UV
3. Smith G, Ribboston SH, Comrie MM, et al. Regulation of cutaneous drug-meta-
bolizing enzymes and cytoprotective gene expression by topical drugs in human
4. Kato S, Bowman ED, Harrington AM, Blomeke B, Shields PG. Human lung car-
cinogen-DNA adduct levels mediated by genetic polymorphisms in vivo. J Natl
5. Soderkvist P, Ahmadi A, Akerback A, Axelson O, Flodin U. Glutathione S-trans-
ferase M1 null genotype as a risk modifier for solvent-induced chronic toxic
360–363.

![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.](image_url)

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