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*Genetics and Selection*

# Methylation of the Glutathione-S-Transferase M3 Gene Promoter in Cystic Fibrosis Patients with 1677 delTA Mutation of CFTR Gene

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**Abstract.** Cystic fibrosis (CF) is the most common autosomal recessive disorder in Caucasians with an incidence of approximately 1/3 000 live births. It is caused by mutations in the cystic fibrosis Transmembrane Conductance Regulator (CFTR) gene. Currently there are over 2000 variants of the CFTR gene reported worldwide and the most common mutant allele of CFTR gene is the F508del. The distribution of CFTR mutations in Georgian CF population differs regarding the high frequency of rare mutation c.1545_1546delTA (1677delTA) and the low frequency of the predominant F508del mutation. Variable clinical manifestations of cystic fibrosis disease even among individuals with same genotypes suggest that not only the diversity of mutations in the CFTR gene, but other genetic and environmental modifier factors influence. In this study we analyzed DNA methylation pattern in Glutathione-S-Transferase M3 (GSTM3) Gene promoter in Georgian cystic fibrosis patients with rare 1677 delTA mutation. The study was approved by the Ethic committee of the Tbilisi State Medical University. 18 CF patients and 10 healthy subjects were recruited in this study. Methylation of GSTM3 Gene promoter was assessed using methylation-specific PCR (MSP) method. Our data indicate a statistically significant difference in GSTM3 promoter methylation level between CF patients and control individuals ($p = 0.0302$). The methylation of GSTM3 promoter was detected in 7 CF patients (7/18, 39%), which was significantly higher than in controls (0/10, 0.0%,). In conclusion, assessing GSTM3 promoter methylation could aid in identifying individuals at higher risk of CF complication and tailoring personalized therapeutic strategies.


**Keywords:** DNA methylation, cystic fibrosis, 1677delTA

## Introduction

Cystic fibrosis (CF) is the most common autosomal recessive disorder in Caucasians with an incidence of approximately 1/3 000 live births. It is caused by mutations in the cystic fibrosis Transmembrane Conductance Regulator (CFTR) gene located on

the human chromosome 7 q31.2 [1]. CF is characterized by recurrent pulmonary infections, elevated sweat chloride, pancreatic and hepatic insufficiency, intestinal abnormalities, failure to thrive, diabetes, meconium ileus (MI) and other glandular defects. Chronic airway dysfunction and inflammation are the main cause of morbidity and mortality of patients [2].

Currently there are over 2000 variants of the CFTR gene reported worldwide and the most common mutant allele of CFTR gene is the F508del (Class II) with a prevalence of 30%-70% depending on the ethnic group [3-5]. The distribution of CFTR mutations in Georgian CF population differs regarding the high frequency of rare mutation c.1545_1546delTA (1677delTA) and the low frequency of the predominant F508del mutation [6, 7].

Recently developed potentiators and correctors (or a combination of them) improve clinical outcomes in CF patients with specific mutations [8]. Results from mutation-based therapy (e.g. Lumacaftor, Ivacaftor) is promising, although not sufficient for rare, non-F508del mutations [9].

Cystic fibrosis patients exhibit a wide spectrum of disease severity and progression ranging from a very mild, adult-onset form to extremely severe cases. Variable clinical manifestations of cystic fibrosis disease even among individuals with same genotypes suggest that not only the diversity of mutations in the CFTR gene, but other genetic and environmental modifier factors influence [10]. Previous studies have demonstrated that epigenetic dysregulation, such as aberrant DNA methylation responsible for the integrity of the epithelium and the inflammatory and immune responses [11-14]. In addition, clinical variability including lung and digestive disease can be explained by modifier genes. Marson et al. studied CF severity in association with several modifier genes including polymorphisms in the glutathione S-transferase M1 (GSTM1) gene. GSTM1 involved in detoxification pathways and related to lung cancer risk in an environment-dependent manner and modifies lung disease severity in CF as well. Study showend that, CF severity is associated with polymorphisms in GSH pathways and CFTR mutations [15, 16]. Magalhães and colleagues detected that GSTM3 gene was differentially methylated in CF patients compared with controls and/or in groups of pulmonary severity in nasal epithelial samples [11]. Thus, the methylation status of the GSTM3 promoter holds potential as a biomarker for the diagnosis and prognosis of various diseases. Assessing GSTM3 promoter methylation could aid in identifying individuals at higher risk and tailoring personalized therapeutic strategies.

In this study we analyzed DNA methylation pattern in Glutathione-S-Transferase M3 Gene promoter in Georgian cystic fibrosis patients with rare 1677 delTA mutation.

## Materials and Methods

The study was approved by the Ethic committee of the Tbilisi State Medical University. Informed written consent was obtained from all patient's parents. In this study recruited 18 CF patients (age $3.7 \pm 2.294$), homozygous for the 1677 delTA mutation ($n = 9$) or compound homozygous for the 1677 delTA /p.Phe508del or another null mutation ($n = 9$) and 10 healthy controls with no history of airway disease (age $5.2 \pm 6.324$).

**DNA extraction and quantification.** Whole blood samples were collected in EDTA (5 ml) tubes for DNA extraction. Genomic DNA was extracted from whole blood samples using the QIAamp DNA Mini Kit (Qiagen, US) according to the manufacturer's recommendations. DNA was quantified by fluorometric method using Qubit fluorometer (Thermo Scientific, USA).

**DNA Methylation of GSTM3 promoter.** Methylation of GSTM3 promoter was determined using methylation-specific PCR (MSP) method as previously described [17]. The MSP-amplified region of GSTM3 is from +75 to +218 within a

CpG island in the 5′ region of GSTM3 gene. The schematic representation of the GSTM3 gene is shown in Figure [18]. The bisulfite modification of genomic DNA (500) was performed using the EpiTect Fast Bisulfite Conversion Kit (Qiagen, CA, USA) to convert all unmethylated cytosines to uracils, while leaving methylated cytosines unaffected according to the manufacturer's protocol. Methylated and unmethylated primers specific for GSTM3 promoter were used to amplify the bisulfate-modified DNA samples. Methylation-specific primers (M) were: Sense: 5'CGT ACG GTT TTG TGG AGT C3'; Antisense: 5'TCC GAA CCT TCG AAA ACT AA3'; Unmethylation-specific primers (U): Sense: 5'TTG TGT ATG GTT TTG TGG AGT T3'; Antisense: 5'CTT CCA AAC CTT CAA AAA CTA AA3'. Size Products for both (M and U primers) were144 bp and annealing temperature for M and U primers was 51.5°C. 25 uL of MSP mixture contained Bisulfite modified DNA 100 ng, 1 µmol/L of each primer and 1 U and Luna® Universal qPCR Master Mix (New England Biolabs, MA, USA). PCR condition was as following: initial denaturation for 10 minutes at 95°C, followed by 40 cycles of denaturing at 95°C for 30 seconds, annealing at 51.5°C for 30 seconds, extension at 72°C for 1 minute and a final extension at 72°C for 7 minutes. Water without DNA was used as negative control.

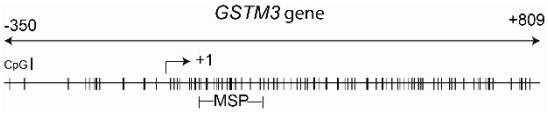


**Fig.** Schematic representation of GSTM3 gene. The transcriptional start site (curved arrow) and CpG site (short vertical lines) were shown. MSP-amplified region of GSTM3 (+75 to +218) is indicated [18].

144 bpPCR products were analyzed by 2.5% agarose gels, stained with ethidium bromide and visualized under UV illumination. We used GelAnalyzer 23.1.1 software to estimate band intensities and compare methylated and unmethylated alleles.

**Data analysis.** Statistical analysis was performed using R Statistical software. The variables were expressed as median. Comparison between groups was performed using Fischer's exact test. the level of significance was set at $p<0.05$.

## Results

We examined the promoter methylation status of GSTM3 gene in 18 CF patients and 10 healthy subjects. Table 2 lists the demographic and relevant clinical characteristics of CF patients and controls.

**Table 1. Demographic and clinical characteristics of CF patients and control subjects**

| Variables | CF (n=18) | Control (n=10) | p value |
|---|---|---|---|
| Mean Age (years) | 3.7 ± 2.294 | 5.2 ± 6.324 | 0.4851 |
| Male (n, %) | 8 (44.4%) | 4 (40.0%) | 1.0000 |
| Female (n, %) | 10 (55.6%) | 6 (60.0%) | 1.0000 |
| Pancreatic Insufficiency (n, %) | 4 (22.2%) | - | |
| Sweat Chloride Level >60mmol/L (n, %) | 18 (100.0%) | - | |
| Pseudomonas Colonization – Yes (n, %) | 5 (27.8%) | - | |
| Failure to Thrive (n, %) | 5 (27.8%) | - | |

The data suggest a statistically significant difference in methylation between CF patients and control individuals ($p = 0.0302$). The methylation of GSTM3 promoter was detected in 7 CF patients (7/18, 39%), which was significantly higher than in controls (0/10, 0.0%,). We used totally methylated (100%) and totally unmethylated DNA (0%) as a known standard (Table 2).

**Table 2. DNA methylation frequencies in CF patients and control subjects**

| Primer pair | Standard | CF (n=18) | Control (n=10) | p value |
|---|---|---|---|---|
| Methylation n(%) | | | | |
| M | 100% | 7 (38.89%) | 0 (0%) | 0.0302 |
| U | 0% | 11 (61%) | 10 (100%) | |

No statistically significant difference was detected in DNA methylation level between 1677 delTA homozygous (n = 9) and compound heterozygous patients (n = 9) (p = 0.921).

## Discussion

The Glutathione-S-Transferase M3 (GSTM3) gene encodes an enzyme crucial for detoxifying electrophilic compounds, thereby protecting cells from oxidative stress [16]. Epigenetic modifications, particularly DNA methylation of the GSTM3 promoter, can significantly influence its expression and have been implicated in various human diseases.

The research indicates that GSTM3 promoter hypermethylation is associated with reduced expression of the gene in colon cancer tissues. This epigenetic alteration may contribute to carcinogenesis by impairing the detoxification of carcinogens, leading to increased DNA damage [19]. In the present study we investigated the DNA methylation profile of GSTM3 gene using blood samples collected from Georgian CF patients with rare1677 delTA mutation. DNA methylation levels were altered in CF patients compared to healthy controls (p=0.0302). Conversely, previous study showed that the mean DNA methylation at GSTM3 was not significantly different in CF and control samples; however, it was associated with CF lung disease severity in NEC samples. Specifically, increased methylation correlated with worse lung function, suggesting that GSTM3 methylation status could serve as a biomarker for disease progression [10].

## Conclusions

Understanding the role of GSTM3 promoter methylation in lung disease pathogenesis offers insights into potential therapeutic interventions aimed at modulating epigenetic marks to restore normal gene function. In summary, assessing GSTM3 promoter methylation could aid in identifying individuals at higher risk of CF complication and tailoring personalized therapeutic strategies.

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# გლუტათიონ-S-ტრანსფერაზა M3 გენის პრომოტორის მეთილირება CFTR გენის 1677 delTA მუტაციის მქონე ცისტური ფიბროზით დაავადებულ პაციენტებში

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ცისტური ფიბროზი (CF) ყველაზე გავრცელებული აუტოსომური რეცესიული დაავადებაა კავკასიოიდურ პოპულაციაში, სიხშირით, დაახლოებით 1 შემთხვევა ყოველ 3000 ახალშობილზე. ის გამოწვეულია ცისტური ფიბროზის ტრანსმემბრანული გამტარობის მარეგულირებელი (CFTR) გენის მუტაციებით. ამჟამად, მსოფლიოში ცნობილია CFTR გენის 2000-ზე მეტი ვარიანტი და CFTR გენის ყველაზე გავრცელებული პათოგენური ალელია F508del. CFTR მუტაციების განაწილება საქართველოს CF პოპულაციაში განსხვავდება და ხასიათდება იშვიათი, c.1545_1546delTA (1677delTA), მუტაციის მაღალი სიხშირით და მსოფლიოში პრევალენტური (F508del) მუტაციის დაბალი სიხშირით. ცისტური ფიბროზის დაავადების კლინიკური ცვლილება ერთი და იმავე გენოტიპის მქონე პირებში იმაზე მიუთითებს, რომ ფენოტიპზე გავლენას ახდენს არა მხოლოდ CFTR გენის მუტაციების მრავალფეროვნება, არამედ სხვა გენეტიკური და გარემო მოდიფიკატორები. წარმოდგენილ კვლევაში გაანალიზებულია გლუტათიონ-S-ტრანსფერაზა M3 (GSTM3) გენის პრომოტორის დნმ-ის მეთილირების დონეები ცისტური ფიბროზის მქონე პაციენტებში იშვიათი 1677 delTA მუტაციით. კვლევა დამტკიცებულია თბილისის სახელმწიფო სამედიცინო უნივერსიტეტის ეთიკის კომიტეტის მიერ. კვლევაში მონაწილეობდა 18 CF პაციენტი და 10 ჯანმრთელი პირი. GSTM3 გენის პრომოტორის მეთილირება შეფასდა სპეციფიკური PCR (MSP) მეთოდით. ჩვენი მონაცემები მიუთითებს, რომ CF პაციენტებში GSTM3 პრომოტორის მეთილირების დონეები სტატისტიკურად მნიშვნელოვნად განსხვავდებოდა საკონტროლო ინდივიდებისგან (p=0.0302). GSTM3 პრომოტორის მეთილირება გამოვლინდა 7 CF პაციენტში (7/18, 39%), რაც მნიშვნელოვნად მაღალი იყო, ვიდრე საკონტროლო ჯგუფის ინდივიდებში (0/10, 0.0%). ამრიგად, GSTM3 პრომოტორის მეთილირების შეფასებამ შეიძლება ხელი შეუწყოს ცისტური ფიბროზის გართულების მაღალი რისკის მქონე პირების იდენტიფიცირებას და პერსონალიზებული თერაპიული სტრატეგიების შემუშავებას.

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