Frequent Methylation of O6-Methylguanine DNA Methyltransferase Gene in Patients with Orofacial Cleft

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Abstract
The O6-methylguanine DNA methyltransferase (MGMT) gene encodes a DNA repair protein and plays a role in embryonic development, including the processes leading to congenital malformations such as orofacial cleft. Methylation at CpG islands of the MGMT gene promoter can affect the regulation of the expression of MGMT or other genes. The aim of this study was to investigate the methylation frequency of the MGMT gene in patients with orofacial cleft.

The methylation status of the MGMT gene promoter was assessed by methylation-specific polymerase chain reaction (MSP) in 24 subjects with orofacial cleft and 24 healthy individuals.

The orofacial cleft group consisted of a significantly (p = 0.04) higher fraction of subjects with fully methylated MGMT gene promoter than the healthy control group.

The finding of the elevated frequency of the methylated MGMT gene suggests a possible risk of reduced DNA repair ability in patients with orofacial cleft.

Keywords: Gene methylation, O6-methylguanine DNA methyltransferase, orofacial cleft


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Introduction
Orofacial cleft is a congenital defect characterized by a cleft in the lip that can be accompanied by a gap in the palate and is caused by an imperfect growth in the orofacial region during the early formation of the fetus.¹ This imperfect growth occurs because of failed fusion of lips and/or the right and left palate, resulting in the formation of the cleft lip and/or the cleft palate.²

The prevalence of birth defects in Southeast Asian countries is quite high, e.g., Indonesia shows a prevalence of 59.3/1000 live births, including orofacial clefts.³ An Indonesian national survey (Riset Kesehatan Dasar) conducted in 2013 reported a prevalence of 0.08% for babies aged 24–59 months with a cleft lip.⁴ In comparison, orofacial clefts affect approximately 15 in every 10,000 births in Europe.⁵

Cleft lip/palate may be associated with syndromic chromosomal and nonsyndromic abnormalities. The nonsyndromic cleft lip/palate is the most common type.⁶ The causes of cleft lip/palate are multifactorial and complex such as environmental, genetic, and epigenetic factors.⁶,⁷ A linkage of these factors may be observed on the cleft lip/palate with or without particular associated syndromic features.⁶

Epigenetics is the study of phenotypic changes or inherited gene expression in the absence of changes in the DNA sequence. A common epigenetic mechanism is DNA methylation.⁶,⁸,¹⁰ In mammals, DNA methylation plays a vital role in genomic molding, X chromosome inactivation, and tissue-specific gene expression.¹¹ DNA methylation determines gene expression, maintains the stability and integrity of DNA, and also determines chromatin modification and mutation progression.¹²

Methylation at the promoter section can cause gene silencing.⁶,⁸ It has been reported that genomic mutations and abnormal epigenetic methylation can contribute to carcinogenesis.¹³

O⁶-Methylguanine DNA methyltransferase (MGMT) is a 24-kDa protein that plays a role in DNA repair through the DNA direct reversal
pathway in mammals. MGMT is expressed throughout cells, including human oocytes, and during early embryonic development. MGMT removes alkyl adducts from the position of O6 guanine to repair a mispair that can cause a point mutation during DNA replication. Although MGMT is not necessarily implicated, genetic risk factors are expected in patients with cleft lip/palate. In humans, the MGMT gene resides at the chromosomal location 10q26.3 and includes one noncoding and four coding exons. The promoter part of the gene has a CpG island with 97 CpG dinucleotides. Methylation primarily occurs at the active sites of the CpG island, i.e., at cytosine that precedes guanine and changes into 5-methylcytosine (5-meC). DNA methylation is a dynamic process that can be carried out by three types of DNA methyltransferases, DNMT1, DNMT3a, and DNMT3b, which act as catalysts to transfer a methyl group from S-adenosylmethionine (SAM) to a cytosine base of DNA in a CG sequence.

The release of O6-meG modification by MGMT is an important step in controlling the checkpoints in cell cycle, proliferation, and differentiation. The expression of the MGMT gene on individual cells or tissue types depends on the variation of several factors, including some stimulus types and promoter regulator elements.

In recent years, several studies have been conducted to investigate the methylation of the MGMT gene involved in DNA repair mechanisms of oral carcinoma cells. Furthermore, past studies have demonstrated that MGMT gene methylation affects congenital malformations in the form of neural tube defects during embryogenesis. Moreover, genetic single nucleotide polymorphic variants of MGMT have been reported to be significantly associated with nonsyndromic orofacial clefts. However, neither genome-wide association studies (GWAS), nor epigenome-wide association studies (EWAS) have so far implicated MGMT in orofacial clefts. Altogether, only a few studies have investigated MGMT gene methylation in patients with orofacial cleft. Therefore, this study was conducted to determine the extent of MGMT gene methylation in subjects with and without orofacial cleft.

Materials and methods

A total of 24 patients with orofacial cleft (18 with cleft lip and 6 with cleft lip and palate) and 24 healthy volunteers were enrolled in this study. From each subject, 1-mL blood samples were collected at the national referral general hospital, with written informed consent, by local doctors under an ethical approval from the Ethics Commission of the Faculty of Dentistry, Universitas Indonesia, No.96/Ethical Clearance/FKGUI/X. 2014. DNA was extracted using previously described techniques.

Methylation status was detected using the bisulfite conversion method, which is the gold standard for this purpose. Bisulfite modification was performed using the EPIjet Bisulfite Conversion Kit (Thermo Scientific) according to the manufacturer’s protocol.

Briefly, the DNA samples (500 ng/1 μL) were diluted with 20 μL of ddH2O. Then, 60 μL of the modification reagent was added along with a mixture of 0.9 mL of ddH2O, 200 μL of modification solution I, and 60 μL of modification solution II into the DNA solution. DNA denaturation was performed at 98°C for 30 min, followed by cooling at 40°C for 30 min. After centrifugation, the liquid at the bottom of the collection tube was removed and the microcolumn in the collection tube was replaced, followed by addition of 100 μL of diluted wash buffer with ethanol, after which the centrifugation and liquid removal steps were repeated. Then, 100 μL of diluted desulphonation buffer was added, and the solution was allowed to stand for 20 min at room temperature, followed by repetition of centrifugation and liquid removal. Again, 100 μL of diluted wash buffer with ethanol was added, and centrifugation and liquid removal were repeated twice. Finally, the DNA was eluted with 5 μL of elution buffer by centrifugation as described above. The bisulfite-treated DNA was amplified by methylation-specific polymerase chain reaction (MSP) using specific methylated and unmethylated template detection primer sets for the MGMT gene. The primer sequences for the methylated template were forward: 5′-TTT CGA CGT TCG TAG GTT TTC GC-3′ and reverse: 5′-GCA CTC TTC CGA AAA CGA AAC G-3′, and those for the unmethylated template were forward: 5′-TTT GTG TTT TGA TGG TTT TAG GTT TTT GTT GC-3′ and reverse: 5′-AAC TCC ACA CTC TTC CAA AAA CAA AAC A-3′.
The MSP was performed in a reaction mixture containing 9 µL of DNA polymerase master mix (Taq PCR Master Mix, Biomix), 2 µL of primary MGMT methylated template detection primer set or unmethylated template detection primer set (10 pmol/each), 6 µL of ddH2O, and 1 µL of bisulfite-treated DNA. DNA amplification was performed with initial denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 45 s, elongation at 72°C for 45 s, and a final extension step at 72°C for 10 min.

The MSP results were identified by electrophoresis on a 1.5% agarose gel (Fermentas) at 80 V/400 mA for 45 min. The amplicons were visualized by GelDoc under UV light after staining with GelRed™ (Nucleic Acid Gel Stain, Biotium). The outcome was interpreted to imply the fully methylated (MM) status of MGMT promoter when it demonstrated only the methylated band and the partially methylated (MU) status when both methylated and unmethylated bands appeared. The fully unmethylated status (UU) would demonstrate only the unmethylated band. The results between the test and control groups were compared using Fisher’s exact test for statistical analysis, assuming the significance at p < 0.05.

Results

Figures 1 and 2 depict examples of the evaluated methylation status of the MGMT promoter, as indicated by MSP. The distribution of subjects with orofacial cleft, also the subgroups of cleft lip or cleft lip and palate, compared with the control group according to the methylation status is presented in Table 1. None of the patients demonstrated the fully unmethylated status. In the group with orofacial cleft, patients with the fully methylated status represented the cleft lip, except for one subject with a cleft lip and palate. In contrast, those with the partially methylated status were more evenly distributed between subjects with cleft lip and those with cleft lip and palate. Overall, the fully methylated status was not uncommon in the group with orofacial cleft, although it appeared at a lower frequency than the partially methylated status. The difference was obvious in the control group that included only patients with the partially methylated status of the MGMT promoter. Due to the small sample size, the subgroups of cleft lip and cleft lip and palate were grouped together to compare only the groups with orofacial cleft and the control group in the statistical analysis. The results of the Fisher’s exact test (Table 2) revealed a significantly higher fraction of subjects with the fully methylated MGMT promoter in the group with orofacial cleft than in the group of healthy control subjects (p = 0.04).
The results of this study are consistent with those reported previously among Chinese subjects, suggesting that *MGMT* gene hypomethylation is associated with congenital malformations such as neural tube defects. However, the level of methylation must be determined in more detail and using a higher number of samples. In addition, these results represent preliminary findings on the effects of orofacial cleft as a congenital malformation and its relationship with the ability of DNA repair mechanisms through the epigenetic modification of the *MGMT* gene promoter. It is necessary to extend the scope of research into further details in the methylated CpG sites of the promoter and to a larger number of samples.

**Conclusions**

All individuals with and without orofacial cleft had some degree of methylation of the *MGMT* promoter. However, the orofacial cleft group had a significantly higher fraction (p = 0.04) of subjects with the fully methylated status than the group of healthy controls that had none. This finding suggests a possible risk of reduced DNA repair ability in subjects with orofacial cleft. A limitation of the study is the modest sample size. A suggested extension should also cover further details in the methylated CpG sites of the promoter.

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**Declaration of Interest**

The authors declare that they have no competing interests.

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