Distribution of E-cadherin 1 (CDH1) promoter methylation in patients with orofacial cleft

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Abstract. Methylation in the promoter region of the E-cadherin 1 gene, CDH1, which plays an important role in cell-to-cell adhesion and craniofacial morphogenesis, potentially results in gene silencing. The present study aimed to assess the methylation status of CDH1 in Indonesian patients with orofacial clefts as well as the potential association between the methylated gene and cleft occurrence. A total of 24 DNA samples from patients with orofacial cleft and 24 control samples from non-cleft individuals were analyzed using methylation-specific PCR after bisulfite treatment to modify the DNA. The CDH1 methylation status of the samples was determined using electrophoresis. In Indonesian patients with orofacial cleft, 5 (20.83%) samples were fully methylated, 19 (79.17%) were partially methylated, and none were fully unmethylated. In contrast, all control samples were partially unmethylated. However, there was no statistically significant difference in the CDH1 methylation status between patients with orofacial cleft and non-cleft individuals (p > 0.05). Methylation of the CDH1 gene promoter was observed in patients with orofacial cleft. However, there was no significant difference in the frequency of CDH1 promoter methylation between patients with orofacial cleft and non-cleft individuals (p > 0.05).

1. Introduction

Orofacial clefts (OFCs) are congenital disorders manifested as a gap in the orofacial area. Clefts can be categorized into palatal clefts and lip clefts with or without palatal clefts. A palatal cleft is defined as the failure in fusion of the palate during development, forming a depression or a fissure. However, lip clefts result from the fusion failure of the maxillary and nasal median processes, resulting in depressions or fissures [1-3]. OFCs can result in deformities at various levels, from indentation of only the upper lip up to the ala of nose. OFCs can also increase the frequency of dental anomalies [4]. Patients with OFCs experience psychological issues and many other complications such as speech disturbances and trouble with swallowing. Babies may face difficulties in consuming food, leading to infant malnutrition. Such complications require a multidisciplinary approach for holistic treatment from birth until adulthood [5].

OFCs are the most common developmental craniofacial anomalies, and its incidence varies according to geography, ethnic origin, and socioeconomic status. According to the Indonesian Basic Health Research (2013), the frequency of cleft lips in Indonesia is 0.08% [6]. This prevalence is significantly lower than that reported by a corresponding 2007 study wherein the occurrence was 0.2%
In the United States, the prevalence of cleft lips with or without palatal clefts is 4.8–17.47 per 10000 births and 2.8–13.45 per 10000 births for cleft palates [2]. According to ethnicity, the prevalence of OFCs is highest among Asians (14 per 10000 births) and native Americans [8]. Caucasians exhibit a prevalence of 10 per 10000 births, and African Americans (populations of sub-Saharan origin) have the lowest prevalence at approximately 4 per 10000 births [1].

The etiology of OFCs remains unestablished; however, it is evident that the disease is of a complex and multifactorial nature. One of the potential causes is epigenetic change, a mechanism of altering phenotype without varying genotype. Epigenetic changes can occur through DNA methylation and histone modification. DNA methylation in the promoter region of a gene is capable of influencing its expression and even causing gene silencing [9-11].

CDH1 is a tumor suppressor gene that plays a role in cell-to-cell adhesion. CDH1 codes for the E-cadherin (epithelial cadherin) protein, a member of the cadherin superfamily that is also a type I transmembrane glycoprotein [12]. Bronchud et al. (2000) and Toyooka et al. (2002) have shown that E-cadherin is involved in morphogenesis and maintenance of differentiated phenotypes [13]. It is important in intercellular junction complex formation as well as the adhesion of epithelial cells and is also believed to play an important role in critical stages of lip and palatal formation. Frebourg et al. (2006) found that CDH1 is highly expressed during the 4th and 5th week of development in the frontonasal process and the 6th week of development in the medial and lateral nasal processes [15]. This study also implied a positive association between OFCs and CHD1 mutations [15,16].

Mutations in CDH1 lead to changes in gene expression, resulting in potential inactivation [14] and DNA methylation and, just like gene mutation, also have the potential to alter gene expression [10]. Therefore CDH1, which participates in craniofacial morphogenesis, is also potentially associated with the occurrence of OFCs.

Epigenetic mechanisms are involved in a wide range of important neurobiological processes such as neurogenesis and brain development, neuronal activity, learning, memory, and circadian rhythm. Disruption of these processes can play a profound role in health and disease, and several epidemiological and clinical features of disease can involve contributions from epigenetic phenomena [17]. To date, there is little information to clarify the association between CDH1 methylation and the occurrence of OFCs. The present study aimed to identify the methylation status of CDH1 in Indonesian patients with OFCs to determine whether it could be one of the epigenetic modification mechanisms involved in cleft formation.

2. Methods
2.1 Samples
This research collected 24 samples of DNA extracts from Indonesian patients with OFCs and 24 samples from Indonesians without clefts. The extracts were stored and frozen at −20 °C in the Oral Biology Laboratory, Faculty of Dentistry, University of Indonesia.

2.2 Sex characteristics of research subjects
From the secondary data, the test group (patients with OFCs) included 10 men (41.7%) and 14 women (58.3%) and the control group comprised 22 men (91.7%) and 2 women (8.3%).

2.3 DNA modification by sodium bisulfite
DNA modification was performed using an EPIJet Bisulfite Conversion Kit (Thermo Scientific) following Protocol B according to the manufacturer’s instructions. The purpose of the DNA modification is to convert cytosine to uracil on unmethylated DNA, whereas the cytosine residues on methylated genes will not get converted.

2.4 Methylation-specific PCR (MSP)
Methylated forward and reverse ECAD as well as forward and reverse unmethylated ECAD primers were used in this study. The sequence of the methylated forward ECAD was 5-
The first step was to prepare a PCR mix for both methylated and unmethylated genes. The PCR mix for the methylated gene comprised 10 µL KAPA 2G Fast ReadyMix + dye (2X), 1µL of methylated forward ECAD primer, 1µL methylated reverse ECAD primer, 7µL ddH₂O, and 1µL of DNA sample, giving a total of 20µL.

MSP was performed under the following conditions: initial denaturation at 95 °C for 5 min; amplification for 32 cycles with denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s; and final extension at 72°C for 10 min.

The PCR products were then analyzed using a 2.5% agarose gel stained with GelRed™ Nucleic Acid Gel Stain. Electrophoresis results were captured with a Gel Doc (Bio-Rad) using Quantity One software.

2.5 Statistical analysis
Data were statistically analyzed using a non-parametric Fischer’s exact test assuming significance at \( p < 0.05 \). The Statistical Program for Social Science version 21 software was used.

2.6 Ethical approval
This research was approved by the Ethics Commission of the Faculty of Dentistry, University of Indonesia (Number: 97/Ethical Clearance/FKGUI/X/2014) since October 1, 2014.

3. Results
A total of 48 subjects were divided in two groups; the test group comprised 24 male and female patients with OFCs, and the control group comprised 24 healthy individuals without any history of clefts. The test group included 10 male patients (41.7%) and 14 female patients (58.3%) with OFCs aged between 5 and 53 years. All participants belonged to various indigenous ethnic groups of Indonesia. The control group comprised 22 males (91.7%) and 2 females (8.3%) without clefts.

After modification with bisulfite, DNA was amplified using methylation-specific polymerase chain reaction (MSP) using specific primers for methylated (M) and unmethylated (U) DNA and each sample with both methylated and unmethylated primer sets. Each pair of reactions for each patient was electrophoresed in parallel and was assigned one methylated (M) and one unmethylated (U) lane.

The presence of a band in the M lane but not in the U lane was interpreted as fully methylated result. Conversely, if the band was visible in the U lane but not in the M lane, the result was interpreted as fully unmethylated. If bands appeared in both lanes, the result was interpreted as partial methylation.

![Figure 1. Fully methylated methylation-specific PCR result.](image-url)
Figure 1 shows a fully methylated MSP result with a clear band in the M lane and no corresponding band in the U lane.

![Figure 1](image1.png)

Figure 2. Partially Methylated and fully methylated methylation-specific PCR results.

Figure 2 shows results from three samples (lanes 2–3, 4–5, and 6–7) that were partially methylated as indicated by bands in lanes 2–7. In contrast, in the last two lanes, a band appeared only in lane 8 and not in lane 9, denoting a fully methylated status.

Table 1 illustrates the distribution of the CDH1 methylation status and allele frequency in the OFC test and control groups. The OFC test group was dominated by partially methylated samples [19/24 (79.17%)]. Only five samples (20.83%) in the OFC test group were fully methylated. In the OFC test group, 60.42% of alleles were methylated and 39.58% were unmethylated. In contrast, 100% of alleles in the control group were partially methylated, giving allele percentages of 50% for both methylated and unmethylated alleles. Thus, partially methylated samples dominated in both test and control groups. Interestingly, fully unmethylated alleles were not observed in any of the samples.

Table 1. Methylation status distribution and allele frequency of orofacial cleft (OFC) Group and Control Group.

| Sample Group | N | Methylation Status (%) | Allele Frequency (%) |
|--------------|---|------------------------|----------------------|
|              |   | MM                     | MU                   | UU                   | M                     | U                     |
| OFC          | 24| 5 (20.83%)             | 19 (79.17%)          | 0                    | 29 (60.42%)           | 19 (39.58%)           |
| Control      | 24| 0 (100%)               | 24 (50%)             | 0                    | 24 (50%)              | 24 (50%)              |

MM: fully methylated  
MU: partially methylated  
UU: fully unmethylated  
OFC: orofacial cleft

Fischer’s exact test showed no significant difference ($P = 0.05$; Table 2) between the OFC and control groups, indicating that there is no significant difference between CDH1 methylation in patients with orofacial cleft and non-cleft individuals.
Table 2. Results from fischer’s exact test on methylation status of the OFC Group.

| Category          | Group      | OFC | Control |
|-------------------|------------|-----|---------|
| Fully methylated  | MM         | 5   | 0       |
| Partially methylated | MU       | 19  | 24      |
| Fully unmethylated | UU        | 0   | 0       |
| Total             |            | 24  | 24      |

MM: fully methylated
MU: partially methylated
UU: fully unmethylated
OFC: orofacial cleft

4. Discussion

OFCs are among of the most common congenital diseases. Although many studies have been conducted, their etiology remains uncertain and is multifactorial. Clefts can occur owing to genetic factors, such as mutation and polymorphism; environmental factors; or a combination of both factors. This research attempted to elucidate an association between cleft occurrence and epigenetic mechanisms, specifically CDH1 methylation. CDH1 is a tumor suppressor gene that codes for the E-cadherin protein, which plays an important role in intercellular adhesion and craniofacial morphogenesis.

Genetic variation and gene mutation may result in changes in gene expression. These changes include repression of gene expression, overexpression, or gene silencing. Methylation in the promoter region of a gene can potentially result in gene silencing, and DNA methylation is thought to be a mechanism that can turn genes on and off during development [9,10]. Therefore, CDH1 methylation itself can result in the disruption of intercellular adhesion systems and can hamper normal craniofacial morphogenesis.

Previous studies have established a relationship between OFCs and cancer. This is supported by the discovery of cleft formation in individuals and families that experience hereditary diffuse gastric cancer [15-19]. Taking this into consideration, it was proposed that both diseases might have the same etiology [18]. Other studies have also demonstrated that genes related to OFCs are involved in cancer, resulting in a hypothesis that the same genes involved in embryonic development later become involved in cancer development [20].

Frebourg et al. demonstrated an association between CDH1 gene mutation and cleft formation [15]. A study by Zhang and Song (2011) on the Han ethnics of China and another by Rafighdoost et al. (2013) on an Iranian population claimed that genetic variations in CDH1 contributes to an increased risk of cleft incidence [18-21]. The E-cadherin protein coded by CDH1 is expressed at a critical moment of lip and palate development [15-19] and Hozyasz et al. (2014) showed that E-cadherin is expressed in epithelial tissues in the palate during and after fusion [18].

The present study was performed to assess the distribution pattern of methylation in the promoter of the CDH1 gene from 24 DNA samples of patients with OFCs, comprising 4 CLP and 20 CL samples. As a control, 24 DNA samples from healthy subjects were used. Methylation was detected using the gold standard method, i.e., MSP combined with bisulfite treatment. Bisulfite treatment converts every cytosine base in the DNA sample to uracil if the DNA is not methylated. However, methylated cytosines are not altered by bisulfite. This method is specific because bisulfite treatment can distinguish between methylated genes and unmethylated ones, thus addressing a specific primer for the methylated and unmethylated genes. From this technique, three results can be obtained: fully methylated (MM), partially methylated (MU), and fully unmethylated (UU). In this study, only full methylation and partial methylation were found; most of the OFC samples were partially methylated (79.17%; 19 out of 24), whereas the remaining five samples were fully methylated; no samples was fully unmethylated.
This study would benefit from the addition of more samples and taking into account the representation of various races and ethnicities in Indonesia to better represent the population. Parents, mainly mothers, of patients with OFCs should also be included as research subjects to investigate the possible existence of epigenetic inheritance mechanisms from parents that might increase the risk of cleft formation. Such information can facilitate early detection of the risk of cleft formation. Finally, further research should be conducted to assess the external factors that might influence epigenetic mechanisms and have the potential to increase the risk of cleft formation.

5. Conclusion
Our findings indicate that methylation of the CDH1 gene promoter occurs in Indonesian patients with OFCs. However, there is no statistical difference in CDH1 promoter methylation between patients with OFCs and non-cleft individuals ($p > 0.05$).

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Conflict Of Interest Statement
The authors state that there are no conflicts of interest regarding the publication of this article.

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