Original Article

The Densitometric Analysis of Protein Pattern in Cleft Lip and Palate Patients

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ABSTRACT

Objectives: Cleft lip and palate (CLP) belongs to the congenital anomaly that is clinically seen as cleft in lip, alveolar bone, palate, and nasal septum. The patients suffer from esthetic and various functional defects. CLP is resulted from impaired palatogenesis during the embryonic phase. The etiology of CLP is influenced by genetic, environmental, and combination of both. According to the literature, CLP is highly associated with defect in interferon regulatory factor 6 (IRF6) and poliovirus receptor-like (PVRL1) genes. The present study aimed to investigate the total protein profile and to identify protein IRF6 and PVRL1 in plasma of CLP patients.
Materials and Methods: Dot-Blot analysis was performed to identify protein target of IRF6 and PVRL1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed in gel concentration 12% using plasma of CLP patients, their parents, and control population. The gels were stained by Coomassie blue afterward. Gels were analyzed through ImageLab 5.2.1 software.
Results: The intensity of major bands in CLP patients was darker than control group, but remains similar to the parents group. The target protein IRF6 and PVRL1 were positively identified through Dot-Blot. Retardation factor value was significantly different in major bands of CLP patients compared to control group.
Conclusion: There pattern of protein profile in CLP patients was different compared to non-CLP.

KEYWORDS: *Cleft lip and palate, interferon regulatory factor 6, poliovirus receptor-like, sodium dodecyl sulfate-polyacrylamide gel electrophoresis*

INTRODUCTION

Orofacial cleft is considered as congenital anomaly with multifactorial etiology. The term orofacial cleft refers to cleft in either lip, palate, or both cleft lip and palate (CLP). The etiology of the cleft is multifactorial, influenced by genetic and environmental factors.[1] CLP occurs more frequently in Asian and Native American populations compared to Caucasians and Africans, with the prevalence of 1/700 live births worldwide.[2] The prevalence of CLP in Indonesia is 0,2%.[3]

CLP is considered as the most common facial birth defect.[4] It resulted from impaired lip and palate formation between 4th and 12th–13th weeks of life.[1] The patients generally suffer from problems related to mastication, recurrent ear infection, phonetic, and malocclusion. Thus, multidiscipline treatments by obstetricians, pediatricians, plastic surgeons, and orthodontists are required.[5]

The etiology of CLP could be either genetic or environmental, such as infection during pregnancy that affects palate development, maternal age (older than 40 years), nutrition deficiency, teratogenic exposure, and smoking.[2,6] The genetic factors of CLP are affected by the expression of various genes that involve in the palate development, such as: Muscle segment

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homeobox 1 (MSX1);[7] T-box transcription factor 22;[8] poliovirus receptor-like 1 (PVRL1);[9] tumor protein 63, fibroblast growth factor receptor 1;[10,11] MSX1; and interferon regulatory factor 6 (IRF6).[7] It was reported that PVRL1 and IRF6 play a significant role in the development of CLP.[12]

PVRL1 encodes protein nectin-1 which involves in epithelial adherence. The fusion of palatal shelves occurs after epithelial fusion of palate during embryonic phase. The process is highly influenced by PVRL1. The defect of PVRL1 is highly associated with CLP.[9] IRF6 is classified as transcription factor that plays roles in orofacial development.[13]

The genetic analysis may provide molecular profiles and changes in DNA, but the protein function, protein activity, and posttranslation modification could be obtained through proteomic analysis. The protein encoded by the genes through translation, posttranslation, and posttranscription may affect directly or indirectly to various biological processes in humans. Therefore, proteomic analysis is needed to support genetic analysis.[14] To the best of our knowledge, there is no study on such studies in Indonesia. Most of the studies related to CLP in Indonesia are based on the clinical aspect. Thus, the present study was aimed to investigate the total protein profile of CLP patients and to identify the protein PVRL1 and IRF6 in CLP patients.

MATERIALS AND METHODS

This is a case–control study with accidental sampling. The study participants were recruited from Surabaya CLP Center. The participants consist of CLP patients (age range: 5–16 years old), their parents, and non-CLP populations as control group. The control group participants matched by age to the CLP group who did not have orofacial clefts, and no family history of clefts. There were 7 ml of peripheral blood obtained from all participants. The blood was centrifuged to obtain plasma and freezed at −80°C for further analysis.

They were 5 µl of each ×10 dilluted samples used to perform Dot-Blot technique. Incubation of membrane was carried out for 12 h under 4°C. The nitrocellulose membranes were blocked with 5% skim milk low fat and phosphate buffered saline-tween (PBS-T) and incubated for 30 min. The membranes were washed using PBS. The primary antibody for IRF6 and PVRL1 (Bioss, USA) were used with a ratio of 1:5000 against PBS. The membrane was soaked in 15 ml of antibody solution for 3 h, and membranes were washed with PBS. 500 µ of TMB was added on top of the membrane and incubated for 15 min in dark room. 500 µl of stop solution was added to the membrane. The immuno Dot-Blot result was scanned for documentation.

The collected plasma was ×10 dilluted using PBS ×1 and pH 8. Laemmli buffer was added to the plasma with ratio of 1:1. Samples were heated for 15 s and froze for 10 min afterward. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) technique was performed on 12% separating gel and there were 25 µl of plasma were put into the well. Gels were run using Mini protean tetra (Bio-Rad) with TGS buffer under 200 V for 45 min. Gels were stained with Coomassie brilliant blue and were destained using solution that contains H_2O, methanol, and acetic acid with ratio of 50/40/10. The samples were incubated for at least 1 h until protein bands were visible. The gels were documented using geldoc EZ (Bio-Rad). The analysis of protein bands was performed through ImageLab software 5.2.1 (Bio-Rad Laboratories, California, USA) and data were presented as images.

The study protocol was submitted for ethical approval by The Ethics Committee of Faculty of Dentistry, Airlangga University (305/HRECC.FODM/XII/2017). All the objectives, risks, and details of this research were fully explained to children and their parents or legal guardians, and written informed consent was obtained from all participants.

RESULTS

The one-dimensional (1D) SDS-PAGE was performed in all participants as shown in Figure 1. There were distinct protein profiles between CLP patients compared to parents and control group. The major bands with molecular weight (MW) range around 15 kDa, 20–25 kDa, 37–50 kDa, and 150–250 kDa were much darker in CLP group compared to control group. In contrary, there were a similar pattern between CLP group and parents group, despite of the fact that the parents who participated in this study do not suffer from CLP.
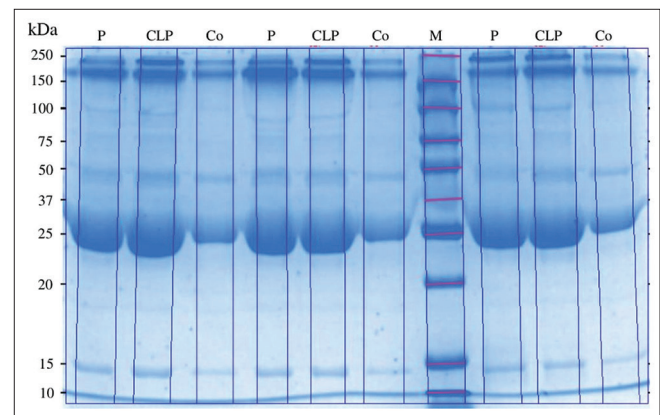

Figure 1: The representative image of analyzed protein pattern by using one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *P* = Parents, CLP = Cleft lip and palate, Co = Control

The protein bands in CLP group tend to have lower MW compared to parents and control group.

The density of major protein bands with MW ranges from 20 kDa to 25 kDa was analyzed as shown in Figure 2, due to their distinct pattern compared to other bands. In CLP group, the intensity was higher, whereas the lowest intensity was found in control group. The data were analyzed using paired *t*-test to compare each group. There was a significant difference of intensity in bands 20 to 25 kDa of CLP group compared to control group ($P = 0.00$). In contrary, CLP and parents group provides insignificant result ($P = 0.35$), with $\alpha = 0.05$. According to MW analysis, the protein in CLP group has lower MW than in parents and control groups as shown in Table 1.

Dot-Blot technique showed positive results for protein target PVRL1 and IRF6 in all groups as shown in Figure 3. The positive results mean that protein PVRL1 and IRF6 were expressed in samples, and SDS-PAGE could be performed for further analysis.

DISCUSSION

The proteins are involved in determining cell structure, biological functions of individuals, and phenotype. Researches trend to explore more of proteomic-based analysis of cells, tissues, and organisms.[15] The

SDS-PAGE is a technique which aims to identify proteins according to their molecular weight.[16] The plasma derived from peripheral blood was used in the present study as it is one of the predominant samples used for diagnostic analysis.[17] Software ImageLab 5.2.1 (BioRad) was used to analyze documented gels in this study. The bioinformatic analysis that was done provide more reliable data compared to manually analyzed gels. To the best of our knowledge, there were still few numbers of studies related to SDS-PAGE used bioinformatic analysis.

The bands of mutated and modified proteins might be expressed in different MW compared to their expected MW.[18] Mutated protein tend to migrate easier during SDS-PAGE, while protein modifications inhibit the migration.[19] Such a phenomenon is termed as gel shifting.[20] The probability of gel shifting reaches up to 40% as stated by Shirai *et al*.[21] The gel shifting occurred in the present study. The protein bands ranging from 20 to 25 kDa are located lower compared to similar bands in other groups, indicating that they have the lowest MW and higher values of retardation factors (Rf). The Rf values provide information about relative migration of proteins within gel. It could be estimated that CLP patients undergo distinct biological process that affects their migration and molecular weight, such as protein modification.[22] This data provides further research opportunities about the mutation, and protein modification occurred in CLP patients.

The intensity analysis exhibits that protein bands ranging 20–25 kDa have different levels of intensity in each group. The bands of CLP groups have the highest intensity (>400) compared to similar proteins in other

Table 1: Analysis of major protein bands

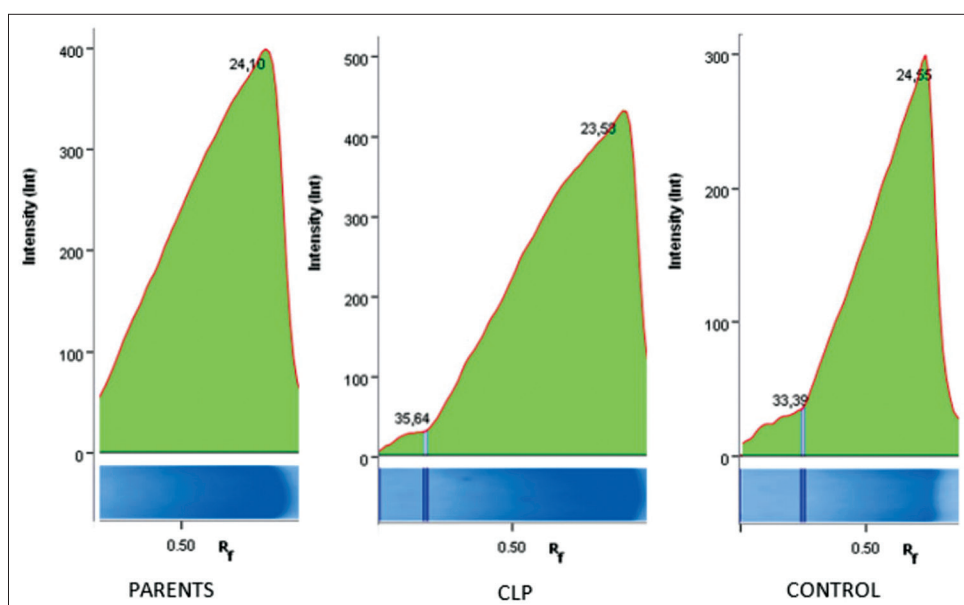| | Bands of 20-25 kDa | | |
	Parents	CLP	Control
Intensity (mean)	408.000	444.000	299.000
Molecular weight (kDa)	24.10	23.58	24.55

CLP=Cleft lip and palate

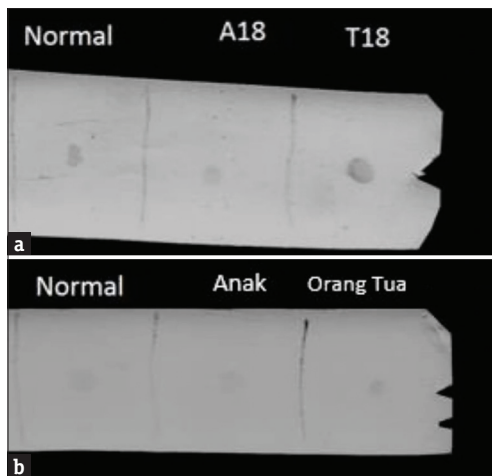**Figure 2:** Densitometric analysis of major protein bands

Figure 3: Positive results of Dot-Blot for protein: Interferon regulatory factor 6 (a) and poliovirus receptor-like 1 (b)

groups. The protein bands in the present study remain relatively constant. The majority appearance of bands in CLP group is darker than other groups, but patterns of parents group tend to look like CLP group even though parents do not possess the clinical appearance of CLP or history of CLP reconstruction surgery. Further analysis is required to identify that proteins and reveals their roles in the pathogenesis of CLP.

The proteins of interest PVRL1 and IRF6 were identified in this study through Dot-Blot technique. It is considered as simplified method of the western blot to detect and required less time-consuming reactions. The electrophoresis procedure was not required, but it provided sensitive result of protein target.[23] A higher number of samples are possible to be analyzed through Dot-Blot, than western blot.[24] The positive result means that protein PVRL1 and IRF6 were expressed in plasma of the participants. It is coherent with the literature stated that PVRL1 and IRF6 play roles in the pathogenesis of CLP.[4,25] However, the Dot-Blot result did not provide the functional analysis of targeted protein and was not possible for quantification. Another weakness of Dot-Blot is that without protein separation, proteins with low MW are masked by high MW proteins on the spot surface. It results in the failure of primary antibody to find its epitope.[24]

IRF6 is a transcription factor that is highly associated with the development of orofacial structure.[13] It is commonly expressed in the medial side of palatal shelves before and during fusion of the palate.[9] PVRL1 or nectin-1 play a role in the adherence of epithelial cells. In the palate development process during embryonic phase, the fusion of palatal shelves occurs after epithelial cells in palate adhere toward each other. That process is influenced by PVRL1. Thus, mutation or defect of PVRL1 is highly

associated with the pathogenesis of CLP.[25] The protein PVRL1 and IRF6 which were positively detected through Dot-Blot give an opportunity for further genomic and proteomic-based analysis to support data obtained from the present study.

The findings of this study are mainly to profile protein bands of CLP patients which provide essential preliminary data for further research related to the advanced and large-scale proteomic analysis of CLP patients, for example, mass spectrometry. This study also aims to identify protein target PVRL1 and IRF6 through immunoblot technique. The results are in line with previous studies stated that PVRL1 and IRF6 are correlated with the incidence of CLP. Moreover, despite the expression of targeted protein could be estimated through the density of bands, it did not give accurate result because there were various other proteins that share same MW as the targeted protein, which could be detected and expressed through 1D SDS-PAGE.

This study lacks participation of patients' family due to geographical and social issue. CLP is considered as fate, aftermath of sinful behavior in the past, and embarrassing for affected family in most of the developing countries,[26,27] including in Indonesia. The negative stigma leads to major obstacle for treatment and conducting research related to CLP, mostly in rural areas, where prenatal diagnosis and patient awareness are low. In spite of the obstacles, this study gives preliminary data for upcoming research, particularly to identify major protein bands and their roles in the pathogenesis of CLP. The studies related to CLP in Indonesia are mainly concern about clinical and social aspects. Proteomic analysis of CLP patients has been carried out in Hungary by Szabo *et al*.,[28] and in China as reported by Zhang *et al*.[29] To the best of our knowledge, there are no previous reports of proteomic analysis of CLP patients in Indonesia. Our intention is to establish proteomic analysis to provide information about upregulated and downregulated protein in CLP patients that could be used as a potential biomarker for predicting the incidence of CLP in high-risk family, or to differentiate whether the etiology of CLP is genetic or environmental.

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Conflicts of interest

There are no conflicts of interest.

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