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The Polymorphism Identification of *Reduced Folate Carrier* 1 (RFC1) in Patients of Non Syndromic Cleft Lip with or Without Cleft Palate in Sumatera Utara

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Abstract
Research Objective:
Cleft lip is the second most frequent congenital anomalies in Indonesia after Down Syndrome with the prevalence among children aged 24-59 months (Agency of Health Research and Development, Indonesian Ministry of Health, 2013). The etiology of non syndromic cleft lip with or without cleft palate has not yet been defined. Some studies have investigated the involvement of genetic and environmental factors (Blanton et al., 2011; Wehby & Murray, 2011). Some genes involved in the folate metabolism have been recently examined in order to discover the genetic factors in the cleft lip etiology. Reduced Folate Carrier 1 (RFC1) protein is not directly involved in the folic acid metabolism but plays a significant role in the intracellular transport of metabolically active 5-methyltetrahydrofolate (MTHF) and maintains the intracellular concentrations of folate. This research intends to identify polymorphism in the Reduced Folate Carrier 1 (RFC1) in the patients of non syndromic cleft lip with or without cleft palate in Sumatera Utara.
Methods:
A number of 46 patients of cleft lip without cleft palate participated in this descriptive study. Subjects' DNAs were extracted from the patients' peripheral leukocytes followed by genotyping procedure on RFC1 A80G gene using Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP).
Results:
Genotype distributions of the subject groups followed Hardy-Weinberg Equilibrium. The mutant genotype frequency (AG+GG) of the subject groups was 63% (29/46).
Conclusion
This research confirms the hypothesis that RFC1 A80G mutant variant holds a greater proportion in the patients *of non syndromic cleft lip with or without cleft palate in Sumatera Utara*
Keywords: NSCLP, RFC1, polymorphism, folic acid

Introduction

Cleft lip is the second most frequent congenital anomalies in Indonesia after Down Syndrome with the prevalence among children aged 24-59 months (Agency of Health Research and Development, Indonesian Ministry of Health, 2013). Cleft lip is divided into 2 big groups, which are cleft lip or without cleft palate and cleft palate only. Then it can classified into the group of non syndromic cleft lip with or without cleft palate (no relationship with other anomalies), syndromic cleft lip with or without cleft palate (part of the known syndrome) and cleft lip with or without cleft palate with *multiple defect* not included the known

syndrome. Piere Robin Sequence (PRS) secara klinis mengelompokkan celah palatum dengan kombinasi mikrognathia, celah palatum bentuk U posterior dan glossoptosis.. (Chango, Emery-Fillon, *et al.*, 2000) The etiology of non syndromic cleft lip with or without cleft palate has not yet been defined. Some studies have investigated the involvement of genetic and environmental factors (Blanton et al., 2011; Wehby & Murray, 2011). The previous study found a relationship between supplementation of folic acid during early pregnancy (≥ 0.4mg/day) with the decreased risk of cleft lip incidence (odds ratio 0.61, and confidence interval 95% 0.39 - 0.96). (Wilcox *et al.*, 2007) In another study, a double blinded Randomized clinical trial was performed by giving folic acid 0.4 mg and 4 mg in women before pregnant until the first trimester and found a decrease of cleft lip incidence. (Wehby *et al.*, 2013) A different result was found in the *United Kingdom* that there was no relationship bêtween the consumption and supplementation of folic acid and the incidence of cleft lip. (Little *et al.*, 2008)

This difference may be affected by the metabolism process of folate including absorption, transportation, modification and interconversion of folate. Some genes involved in the folate metabolism have been recently examined in order to discover the genetic factors in the cleft lip etiology. Reduced Folate Carrier 1 (RFC1) protein is not directly involved in the folic acid metabolism but plays a significant role in the intracellular transport of metabolically active 5-methyltetrahydrofolate (MTHF) and maintains the intracellular concentrations of folate. This research intends to identify polymorphism in the Reduced Folate Carrier 1 (RFC1) in the patients of non syndromic cleft lip with or without cleft palate in Sumatera Utara.

Material and Method

A number of 46 patients of cleft lip without cleft palate participated in this descriptive study. To all the subjects participating in the study, previously were explained the objective, procedure, benefit, risk as a subject in this study and the unpleasant feeling that may be present to the responsible family due to the implementation of the study procedure. All of the subjects who agreed to participate in the study, were asked to sign an informed consent to follow the study. Subjects' DNAs were extracted from the patients' peripheral leukocytes followed by genotyping procedure on RFC1 A80G gene using Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP). Blood samples as much as 3 ml (minimum 1 ml) were collected from the study subjects and were stored in a tube containing anticoagulant, Ethylenediaminitetraacetic acid (EDTA) appropriate to DNA examination. The samples were examined using genotyping RFC1 A80G with the procedure of PCR-RFLP.

Results

Characteristics of study subjects

Table 1. Distribution of Subjects based on the classification of cleft lip

	N	**%**	**CI 95%**
Cleft lip only	4	8.7	7.55 – 9.85
Cleft lip with cleft palate	40	87	86.48 – 87.52
Cleft palate only	2	4.3	3.46 – 5.15
Total	46	100.0	

In this study, the most distribution was in the group of cleft lip subjects with cleft palate, as much as 40 patients (87.0%), and the least was in the group of subjects with cleft palate, only 2 patients (4.3 %).

In this study, most of the subjects were male as much as 29 patients (63 %) compared to 17 patients who were female (37%).

Table 2. Proportion comparison of the diagnosis of cleft lip between Gender

		Diagnosis of Cleft Lip					
		CLO		CLP		CPO	
		N	%	N	%	N	%
Gender	Male	3	75	25	62.5	1	50
	Female	1	25	15	37.5	1	50
Total		4	8.7	40	87	2	4.3

Diagnosis

From the result of study, we can determine the characteristic of subjects based on the diagnosis as follows:

Table 3. Distribution of Subject based on the Diagnosis

Diagnosis	**N**	**%**	**CI 95%**
Right Complete CLP	10	21.7	20.15 – 23.2
Left Complete CLP	20	43.5	41.91 – 45.09
Bilateral Complete CLP	10	21.7	20.15 – 23.2
Right Complete CL	1	2.2	1.58 – 2.82
Left Complete CL	3	6.6	5.48 – 7.52
Cleft Palate	2	4.3	3.46 – 5.15
Total	46	100.0	

CLP = *Cleft Lip and Palate*

CL = *Cleft Lip*

In this study, the most distribution was in the group of subjects diagnosed with *Left Complete Cleft Lip and Palatum* as much as 20 patients (43.5 %), and the least was in the group of subjects diagnosed with *Right Complete Cleft Lip*, just 1 patient (2.2 %).

Distribution of Genotype Variant RFC1 A80G

The result of this study obtained the distribution of Genotype Variant RFC1 A80G in study subjects as follows.

Table 4. Distribution of Genotype Variant RFC1 A80G in study subjects

	N	%	CI 95%
Genotype A/A	17	37.0	35.37 – 38.63
Genotype A/G	21	45.7	44.14 – 47.26
Genotype G/G	8	17.4	15.93 – 18.87

Total	46	100.0	

In this study, the distribution of study subjects based on polymorphism type RFC 1 were most in the variant A/G group, as much as 21 patients (44.7%) and the least in the variant G/G group, 8 patients (17.4%). After classification, we obtained subjects included into Wildtype as much as 17 patients (37%) and mutants, 29 patients (63 %)

Table 5. Classification Distribution of Polymorphism RFC1 in study subjects

	N	**%**	**CI 95%**
Wildtype (AA)	17	37.0	35.37 – 38.63
Mutant (AG+GG)	29	63.0	61.75 – 64.25
Total	46	100.0	

Frequency of Alelle RFC1 A80G in study subjects

The results of study obtained the frequency of alelle RFC1 A80G in study subjects as follows

Table 6. Frequency of Alelle RFC1 A80G in cleft lip patients

	N	**%**	**CI 95%**
Alelle A	55	59.78	59.12 – 60.44
Alelle G	37	40.22	39.41 – 41.03
Total	92	100.0	

In this study, the most frequency was in the group of study subjects with allele A RFC 1, which were 55 alelles (59.78%) compared to the group of subjects with the allele G, 37 alelles (40.22 %).

Discussion

In the population of cleft lip with or without cleft palate, the most diagnosis was *cleft lip and palate* as much as 46%, followed by *cleft palate,* 33% and *isolated cleft lip,* 21% (Hopper, 2013). This was slightly different from the result of this study, with the most diagnosis of *cleft lip and palate* as much as 87%, *isolated cleft lip,* 8.7 % and *cleft palate*, 4.3%. The proportion difference occurred because this study the collection of samples was done by *consecutive sampling* only for 3 months (April to June 2017). In this study, we found *Left Complete* CLP as much as 43.5%, *Right Complete* CLP, 21.7% and *Bilateral Complete* CLP, 21.7%. This was not too different from the statement by Hopper et al, 2013; that *unilateral cleft* was nine times more frequent than *bilateral cleft* and occurred two times more often on the left side than the right side.

Polymorphism RFC -1

The Result of Electrophoresis PCR-RFLP Polymorphism RFC 1

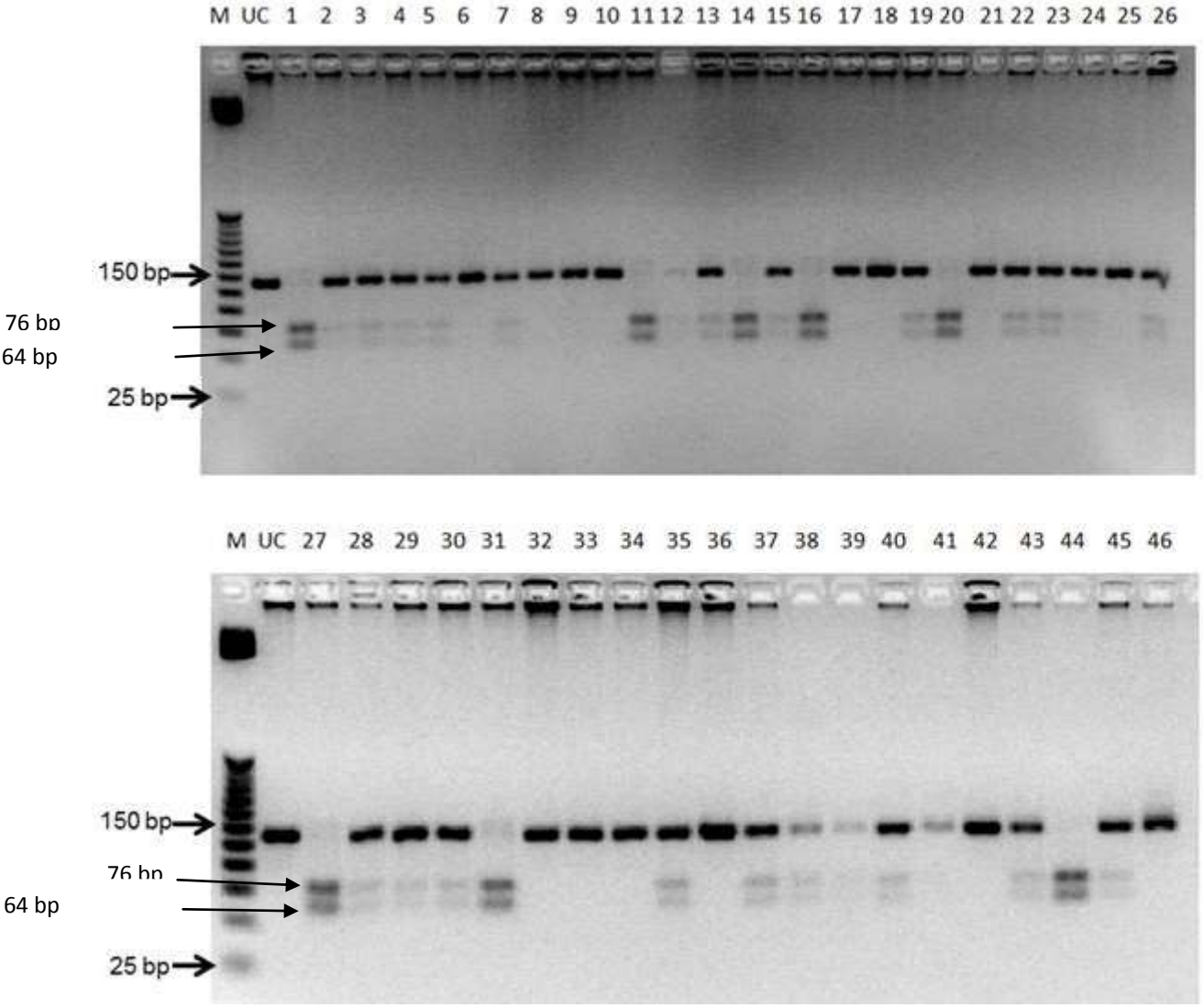

Figure 1 The Result of Electrophoresis, Product of PCR-RFLP Analysis of SNP RFC-1 restricted by enzyme HaeII from each subject.

Figure Notes: Lane M is a DNA Ladder, Lane UC is a product of PCR RFC 1 not cleaved by enzyme HaeII, Lane 1-46 are samples with no. 1- 46. At fragment 140bp it is visible that Alel A and alel G were seen at 76bp and 64bp.

Based on the distribution of gender, according to Hopper *et al*,2013; male were dominant in the population of cleft lip and palate, where *isolated cleft palate* occurred more often in female. The statement above is suitable with result of this study that obtained data of patients diagnosed with *Cleft Lip Palate* (CLP) 62.5% subjects were male and 37.5% were female; and *isolated cleft palate* were found only in 2 cases from 46 study subjects with the proportion of male and female, both 50%.

This study obtained the data of study subjects having the variant AA sejumlah 17 orang (37%), AG as much as 21 patients (45,7%) and GG, 8 patients (17,4%), this data was slightly different from the data found in India by Lakkakula *et al,2015;* subjects having the variant AA, 18 patients (12.7%), AG, 64 patients (45.1%) and GG, 60 patients (42.2%). Other studies about polymorphism RFC1 in non syndromic cleft lip patients was performed in Italy by Girardi *et al.*, 2017 obtained the data of Alelle A as much as 56% and

alelle G, 44%, the data above was similar to this study data which found allele A as much as 59,78% and allele G, 40.22%. But the proportion was different with the study by Lakkakula *et al*,2015; whoo found the alelle A, 35.2% and Alelle G, 64.8%.

The classification of variants to *wildtype* and mutant in this study obtained a proportion of subjects with mutant were more compared to the subjects of *wildtype* with the details of *wildtype* as much as 17 patients (37%) and mutant, 29 patients (63%). This is consistent to the data of Lijun Pei *et al,2006;* whom found subjects with *wildtype* as much as 18 patients (22%) and mutant, 64 patients (78%), as well as consistent to the study by Lakkakula *et al,2015;* with *wildtype*, 18 patients (12.7%) and mutant, 124 (87.4%)

Conclusion

Patients with cleft lip brought the Variant AA as much as 37%, AG 45.7% and GG 17.4%; variant *Wildtype*, 37% and Mutant, 63%. The frequency of Alelle A in study subjects was 59.78% and Alelle G, 48.22%.

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