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Research Article

Expression of TGF-α and TGF-β3 Proteins in the Incidence of Cleft Lip and Palate in East Nusa Tenggara

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Abstract

A cleft lip (CL) is a gap in the upper lip caused by unconnected tissues before birth, often found alongside a cleft palate (CP). In Indonesia, the prevalence of orofacial clefting is around 1:1,596. The incidence is multifactorial and polygenic. This study aimed to assess the expression of TGF-α and TGF-β3 proteins in endemic clefts of East Nusa Tenggara. This study used a cross-sectional method to analyze lip epithelial tissue from patients with nonsyndromic CL/P. The research was conducted at the Bioscience Laboratory, Central Laboratory of Life Sciences, and Biomedical Laboratory, Universitas Brawijaya. This research started in April 2023 until October 2023. The study involved laboratory preparation, paraffin block and preparate making, and immunofluorescence staining. The tissues were infiltrated with soft paraffin, trimmed, and thinned. The data was analyzed using Image J with a threshold of 20-100 and an independent t-test. TGF-α protein expression showed significant differences between East Nusa Tenggara and control, but no significant d ifference in TG F-β3 ex pression. Th is research showed that TGF-α expression in the East Nusa Tenggara region is lower than control. This incidence may be due to inbreeding. Low TGF-β3 expression in patients with cleft palate only (CPO) is due to the complex development of the palate. The study reveals that low expression of TGF-α and TGF-β3 proteins in the East Nusa Tenggara region contributes to cleft lip and palate incidence, affecting cell proliferation and palatal shelf adhesion.

Keywords: *cleft lip, cleft palate, proteins, TGF-α, TGF-β3*

Ekspresi Protein TGF-α dan TGF-β3 terhadap Kejadian Bibir Sumbing dan Langit-langit Sumbing di Nusa Tenggara Timur

Abstrak

Bibir sumbing merupakan celah pada bibir atas yang disebabkan oleh jaringan yang tidak terhubung sebelum janin lahir, sering ditemukan bersamaan dengan celah langit-langit. Di Indonesia, prevalensi celah orofasial sekitar 1:1.596. Insidennya bersifat multifaktorial dan poligenik. Penelitian ini bertujuan untuk menilai ekspresi protein TGF-α dan TGF-β3 pada celah bibir dan langit-langit di Nusa Tenggara Timur. Penelitian ini menggunakan cross-sectional method untuk menganalisis jaringan epitel bibir dari pasien bibir sumbing/celah langit-langit nonsindromik. Penelitian dilakukan di Laboratorium Biosains, Laboratorium Sentral Ilmu Hayati, dan Laboratorium Biomedik, Universitas Brawijaya. Penelitian ini dimulai pada bulan April 2023 hingga Oktober 2023. Penelitian ini meliputi persiapan laboratorium, pembuatan blok dan preparat parafin, pewarnaan imunofluoresensi. Jaringan diinfiltrasi dengan parafin lunak, dirapikan, dan ditipiskan. Data dianalisis menggunakan Image J dengan ambang batas 20-100 dan uji t independen. Ekspresi protein TGF-α menunjukkan perbedaan bermakna antara sampel Nusa Tenggara Timur dan kontrol, tetapi tidak terdapat perbedaan bermakna pada ekspresi TGF-β3. Penelitian ini menunjukkan bahwa ekspresi TGF-α di wilayah Nusa Tenggara Timur lebih rendah dibandingkan kontrol. Kejadian ini mungkin disebabkan oleh perkawinan sedarah. Ekspresi TGF-β3 yang rendah pada pasien celah langit-langit disebabkan oleh perkembangan langit-langit yang kompleks. Penelitian ini mengungkap bahwa ekspresi protein TGF-α dan TGF-β3 yang rendah di wilayah Nusa Tenggara Timur berkontribusi terhadap kejadian bibir sumbing dan langit-langit, yang memengaruhi proliferasi sel dan adhesi palatal shelf.

Kata kunci: *celah bibir, celah langit-langit, protein, TGF-α, TGF-β3*

Introduction

A cleft lip (CL) is a gap in the upper lip caused by the tissues that make up the lip not connecting properly before birth. The cleft can occur on either side of the lip or in the center of the lip. Cleft lip is often found together with cleft palate (CLP). Cleft palate (CP) occurs due to incomplete formation of palate tissue during pregnancy.[1] Potential complications of the CLP disorder include speech impairment, hearing, malocclusion, serious psychiatric issues, significant facial deformities, and social handicaps such as impaired suckling and consequent failure to grow.[1] The global incidence of orofacial clefting is 220,000 new cases annually, approximately 1.5 out of 1,000 births. Asian populations hold the highest incidence rate, with 0.82–4.04 per total births. Meanwhile, the African population has the lowest incidence rate of 0,18–1,67 per birth.[2] In Indonesia, cleft lip and palate prevalence is 1:1,596. According to Sjamsudin and Maifara,[3] the percentage of cleft lip is 24.42%, cleft palate is 25.05%, and cleft lip and palate is 50.53%, with the highest frequency in male patients at 55.95%, while for women, it is 44.05%. The CLP tendency is higher in boys, while CP is higher in girls.[4] The incidence of CL is primarily found in low-income communities.[3] The Nusa Tenggara region has one of the fourth-highest poverty rates in Indonesia.[5,6] This condition is one of the factors for the high CLP rate in the East Nusa Tenggara region due to the lack of nutrition, which impacts the embryonic development of a fetus. The incidence of cleft lip and palate in East Nusa Tenggara Province, which is 8.6% or reaches a rate of 6-9 incidents per 1,000 population,[7] has caused East Nusa Tenggara to become a CLP endemic area. Almost all regions of East Nusa Tenggara have CLP incidence rates.[8] Other factors, such as the tradition of inbreeding, are also reasons for the high incidence of CLP in East Nusa Tenggara. The incidence of cleft lip and palate is multifactorial and polygenic, including genetic, environmental, and interaction between them, as well as nutritional (macro-nutrients and micro-nutrients) factors.[8-10] These nutritional deficiencies lead to stunting and failure of palate-lip fusion.[8] Efforts to determine the etiology of CLP as a preventive measure have been made with comprehensive approaches such as epidemiology, phenotyping, genomic studies, and linkage of genes and environmental factors.[11] Research observing the incidence of non-syndromic CL can use the interaction of genes with risk factors. The use of transforming growth factor-A (TGF-a) and transforming growth factor-β-3 (TGF-β3) genes can be an alternative to observing the incidence of CLP because the function of these genes plays an important role in the process of palatal development.[9,12]

These two genes have important functions in the process of craniofacial development. During craniofacial development, TGF-α is expressed at the edge of the mid-epithelium of the fused palate and promotes extracellular matrix synthesis and mesenchymal cell migration towards the palate.[9] TGF-α promotes the migration of mesenchymal cells and the formation of extracellular matrix to ensure the strength of the fused palate during the palate development process. TGF-β3, an important member of the TGF-β superfamily, regulates mesenchymal cell proliferation, extracellular matrix changes, and epithelial shelf attachment by cell differentiation.[13] TGF-β3 is expressed in the medial edge epithelium (MEE) before and after fusion and will disappear at the end of the palate fusion process (week 12-13 of gestation).[14] Deficiency in TGF-β3 and TGF-α expression leads to cleft lip.[15] Therefore, in this study, we assessed the expression of TGF-α and TGF-β3 proteins in endemic clefts of East Nusa Tenggara.

Methods

This study was an analytical observational study using the cross-sectional study design. This study was conducted in April 2023 until October 2023. A total of 30 samples were collected from 30 patients diagnosed with nonsyndromic CL, CP, and CLP. Samples were transferred to 10% NBF for tissue fixation. The sample was then subjected to paraffin block and preparate making at the Anatomical Pathology Laboratory, Faculty of Medicine, Universitas Brawijaya. Immunofluorescence staining was conducted in Biomedical Central Laboratory, Faculty of Medicine, Universitas Brawijaya. The inclusion criteria in this study were patients who were not found to have other congenital diseases and the samples used were leftover lip epithelial tissue of at least ≥5 grams.

Exclusion criteria are patients with other congenital diseases, such as other facial or skeletal malformations and metabolic neurologic disorders. The Ethics Committee of the Faculty of Medicine, Universitas Brawijaya, granted the study ethical approval with the number 115/EC/KEPK-S1-PD/06/2023.

Block Paraffin Preparations

After being cleaned with phosphate-buffered saline (PBS), the tissue samples were left to cure for 24 to 48 hours at room temperature in a 10% formalin solution. Subsequently, the tissues were

cleared with xylol and dehydrated using a succession of alcohol concentrations (70%, 80%, 95%, and 100%). After the tissues were infiltrated with soft paraffin at 48ºC, the paraffin wax was formed, the tissues were then embedded into blocks, and the process was allowed for a day. The paraffin blocks were trimmed to fit the holder and thinned using a rotary microtome to 4–6 µm. The paraffin ribbon was soaked in a 40–45°C bath before adhered to a glass surface using 5% gelatin. The object glass's paraffin was submerged in xylol, rehydrated using an alcoholic series (70%, 80%, 95%, and 100%), and then washed with deionized water.

Immunofluorescent Staining

Staining of preparations with immunofluorescence labels TGF-β3 and TGF-α was carried out by the indirect method. Prepared tissue slides that have been heated at 60°C for 60 minutes were then immersed in xylol solution 2x10 minutes, absolute ethanol 2x10 minutes, 90% ethanol for 5 minutes, 80% ethanol for 5 minutes, 70% ethanol for 5 minutes, and distilled water for 3x5 minutes. After these steps, the preparation was immersed in a chamber containing citrate buffer with pH 6.0 and put into a 95°C water bath for 20 minutes. Slides were washed using PBS 3 times, each for 5 minutes, and then washed with Triton-X 100 PBS with a concentration of 0.1% for 5 minutes. Slide preparation was then incubated with 1% bovine serum albumin (BSA) for 30 minutes at room temperature. Then, the BSA solution was removed, and the slides were dried with a tissue.

The dried slides were incubated with primary antibody (Thermo Scientific MA5-34680 TGF alpha Recombinant Rabbit Monoclonal Antibody (JB58-36)) overnight at 4°C. The slides were washed with PBS 3 times for 5 minutes each. The slide preparation was then labeled with secondary antibodies (Abcam ab6718 Goat Anti-Rabbit IgG H&L (TRITC, Abcam ab6785 Goat Anti-Mouse IgG H&L (FITC)) 1:1,000 for 30 minutes at room temperature under dark conditions. The slides were rewashed with PBS 3 times for 5 minutes each. Then, it was incubated with DAPI dye (Abcam ab228549 DAPI Staining Solution) 1:1,000 for 5 minutes and washed with PBS 3 times for 5 minutes each. The slides were covered with mounting medium and cover glass and then observed on a fluorescence microscope (Olympus IX71).

Data Analysis

Immunofluorescence data analysis was performed with Image J with a threshold of 20–100. The data taken includes data on the area of the stained area. The threshold data used includes data on the area of the region that has been colored. Threshold data analysis is carried out on preparations with 40x magnification. Independent t-test to determine whether there is a significant difference between TGF-α and TGF-β3 levels in East Nusa Tenggara and control. The $p<0.05$ was considered statistically significant. All data were analyzed using SPSS 26.0.

Results

The total number of cleft lip tissue was 30 samples from 30 patients. The samples were leftover lip epithelial tissue from the Sekar Lintas Nusantara Foundation's social service activities with Dr. Saiful Anwar Hospital in several regions in Indonesia (Table 1).

The largest age group was between the ages of <1 year, as many as 12 people (40%), and 2–5 years, as many as 14 people (46.6%). While the smallest age group was between the ages of 6-10 years, as many as 4 people (13.3%). Based on the analysis results of TGF-α protein expression in samples from East Nusa Tenggara and control (Non-East Nusa Tenggara), interesting results were obtained. The protein expression of TGF-α and TGF-β3 was successfully observed using the immunofluorescence technique (Figure 1–2).

The t-test results showed a significant difference between the East Nusa Tenggara and control samples with a p-value of 0.023. Based on these results (Table 2), TGF-α protein expression in the control is more than in the East Nusa Tenggara. The same pattern was also shown in the TGF-β3 variable, where protein expression from the control was greater than the East Nusa Tenggara. However, statistically, the expression of TGF-β3 in the East Nusa Tenggara and control did not significantly differ.

Table 1. Sample Used in This Study

East Nusa Tenggara		Control (Non-East Nusa Tenggara)	
Sample Code	Locality	Sample Code	Locality
KP1	Kupang, East Nusa Tenggara	LU1	Lumajang, East Java
KP2	Kupang, East Nusa Tenggara	LU2	Lumajang, East Java
KP3	Kupang, East Nusa Tenggara	LU3	Lumajang, East Java
KP4	Kupang, East Nusa Tenggara	LU4	Lumajang, East Java
KP5	Kupang, East Nusa Tenggara	LU5	Lumajang, East Java
BO1	Borong, East Nusa Tenggara	P01	Palangkaraya, Central Kalimantan
BO2	Borong, East Nusa Tenggara	P02	Palangkaraya, Central Kalimantan
BO3	Borong, East Nusa Tenggara	P04	Palangkaraya, Central Kalimantan
BO5	Borong, East Nusa Tenggara	P05	Palangkaraya, Central Kalimantan
BO7	Borong, East Nusa Tenggara	P06	Palangkaraya, Central Kalimantan
LR01	Larantuka, East Nusa Tenggara	M03	Manado, North Sulawesi
LR04	Larantuka, East Nusa Tenggara	M04	Manado, North Sulawesi
LR05	Larantuka, East Nusa Tenggara	M07	Manado, North Sulawesi
LR13	Larantuka, East Nusa Tenggara	M08	Manado, North Sulawesi
LR02	Larantuka, East Nusa Tenggara	M10	Manado, North Sulawesi

Discussion

The variables in this study were the expression of TGF-α and TGF-β3 in the incidence of CLP in East Nusa Tenggara. TGF-α showed significant results where the expression level of TGF-α protein in the East Nusa Tenggara is smaller than the control. This could cause the high CLP rate in East Nusa Tenggara, as indicated by the low expression of TGF-α protein. Our result is similar to previous research conducted by Wihastyoko et al[16] which revealed that TGF-α in CL showed decreased expression compared to normal lip. Justification of this result corroborates that the incidence of CLP in the protomalayid race is also influenced by the low expression of TGF-α, which causes a decrease in signaling for cell activation so that cell proliferation, migration, differentiation, and development also decrease. Despite the MAPK pathway, the complexity of cleft events is caused by many factors such as TGF-α, TGF-β1, FGF-2, SVEGFR1, AP-1, p38, ERK-1, and collagen.[2] The low TGF-a in East Nusa Tenggara races compared to control (Non-East Nusa Tenggara) can be caused by genetic factors, whereas in the East Nusa Tenggara, inbreeding still occurs. Inbreeding can cause a lack of genetic diversity and increase the frequency of mutations.[4]

The results of this study are similar to previous research conducted by Chandra et al[17] using cross-sectional analytical methods that discussed the relationship between epidermal growth factor receptor (EGFR) and extracellular signal-regulated protein kinase-1 (ERK-1) proteins in the incidence of cleft lip protomalayid race in East Nusa.

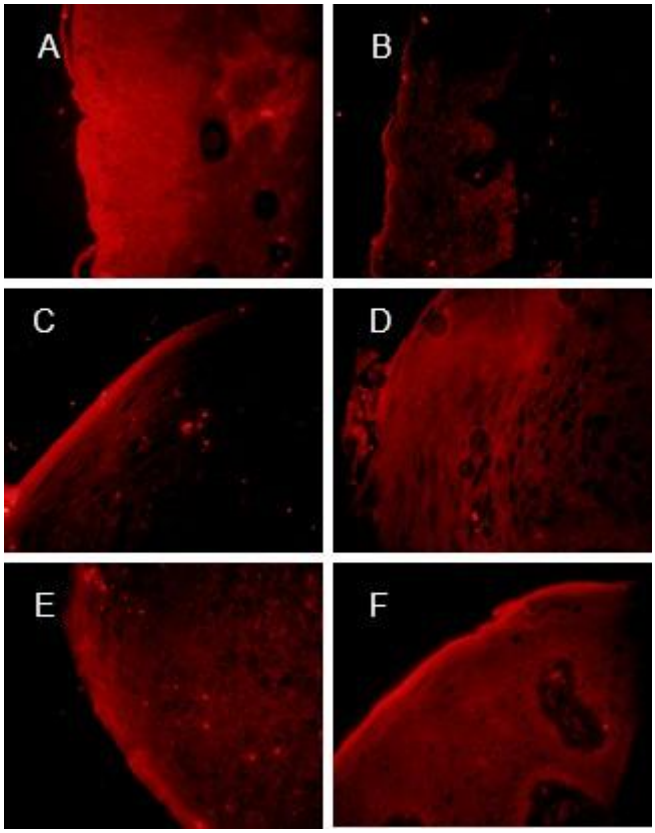

Figure 1. TGF-α Protein Expression (A) Kupang, (B) Borong, (C) Larantuka, (D) Lumajang, (E) Palangkaraya, (F) Manado

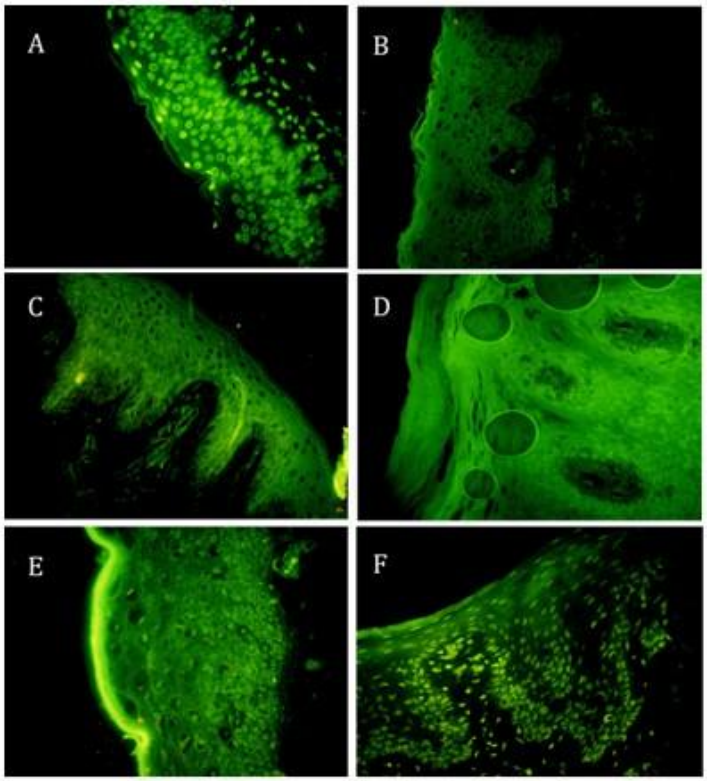

Figure 2. TGF-β3 Protein Expression (A) Kupang, (B) Borong, (C) Larantuka, (D) Lumajang, (E) Palangkaraya, (F) Manado

Table 2. Analyses of Protein Expression

Protein Expression	Locality	Mean	*p-value*
TGF- α	East Nusa Tenggara	11.87	0.023*
	Non-East Nusa Tenggara	22.31	
TGF-β3	East Nusa Tenggara	16.87	0.364
	Non-East Nusa Tenggara	21.01	

This study found that EGFR protein expression affects the proliferation process in cleft lip incidence.[7] TGF-α is a member of the EGF family, where the role of TGF-α is to activate EGFR, which will activate the Ras/Ref/MEK signaling pathway.[16] The signaling pathway activation will stimulate a series of phosphorylations involving the activation of MAPK, ERK-1, and ERK-2. The function of ERK-1 and ERK-2 proteins is for transcriptional regulation of molecules related to proliferation, adhesion, mutation, survival, and migration.[17]

Based on the results of this study, the mean value of TGF-β3 protein in the East Nusa Tenggara group was lower than the control, although the difference was not significant. The low expression of TGF-β3 can cause failure in the process of palatal shelf adhesion. TGF-β3 is required to control mesenchymal cell proliferation, extracellular matrix changes, and epithelial attachment through cell differentiation. During the fusion process, TGF-β3 regulates the epithelial-mesenchymal transformation of MEE cells.[18,19] The MEE cells subsequently disappear from the median line, and the palatal shelves can fuse completely.[20]

TGF-β3 expression is low in cleft palate only (CPO) patients because extracellular matrix (ECM) components and growth factors interact spatially and temporally along a complex chain of events leading to the development of the palate. The palatal portion rapidly rises to a horizontal position over the tongue during orofacial development, attaches to the midline, and eventually fuses. The forces driving palatal elevation also depend on the local accumulation of glycosaminoglycans (GAGs), especially hyaluronan (HA), in the mesenchyme because water absorption of GAGs and HA induces palatal swelling and stiffness. Genetic and environmental variables can cause CPO, including elevation failure, fusion errors, post-fusion shelf rupture, or shelf growth defects.[21]

Conclusion

The conclusion from the results of this study is that the expression of TGF-α and TGF-β3 proteins in the incidence of CLP in the East Nusa Tenggara is lower than the control. Statistically, TGF-α protein expression showed a significant difference, but TGF-β3 protein expression did not significantly differ with the control. Low expression of TGF-α leads to decreased signaling for cell activation, which causes cell proliferation, migration, differentiation, and development also to decrease. Meanwhile, low expression of TGF-β3 can cause failure in the process of palatal shelf adhesion. Thus, the expression of these two proteins is one of the causes of cleft lip and palate incidence in East Nusa Tenggara.

Conflict of Interest

The author declares no conflict of interest.

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