

RESEARCH ARTICLE

The Potency of NTHi *lic1A* Gene as a Biomarker in Determining The Severity of Post-Viral Acute Rhinosinusitis

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Abstract

BACKGROUND: The *lic1A* gene is an important virulence factor for non-typeable *H. influenzae* (NTHi), which allows its translocation from the nasopharynx into the sinonasal cavity and modulates more severe inflammatory processes. This study is aimed for identifying the potential correlation between the NTHi *lic1A* gene expressions and the severity of post-viral acute rhinosinusitis.

METHODS: Sixty patients who were diagnosed with post-viral acute rhinosinusitis, were recruited from an ENT clinic in a referral hospital, in Bandung, West Java, Indonesia. All patients underwent a historical assessment and ENT examination. The nasal specimen was taken from the patient's middle meatal. The NTHi *lic1A* gene expression was detected using Polymerase Chain Reaction (PCR).

RESULTS: We observed that eight patients had the NTHi *lic1A* (+), with a strong correlation toward the dominant symptoms (nasal obstruction and discharge). In addition, the symptom's duration of the NTHi *lic1A* (+) was twice longer than patients with the NTHi *lic1A* (-). Its severity was significantly more different between the two groups ($p=0.034$).

CONCLUSION: Taken together, the presence of the NTHi *lic1A* gene is significantly associated with the severity of the disease and the symptom's duration. Thus, the NTHi *lic1A* gene could potentially be a good marker for assessing the severity of post-viral acute rhinosinusitis cases in the future.

KEYWORDS: *H. influenzae*, rhinosinusitis, nasal obstruction, virulence factors

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Introduction

Acute rhinosinusitis is a common diseases in the community, affecting 6-10% of the outpatients population in several Asian country including Indonesia.(1) Acute rhinosinusitis can be classified into viral rhinosinusitis, that commonly caused by rhinovirus and coronavirus, and post-viral acute rhinosinusitis, a residual mucosal inflammation following a

viral infection that produces ongoing symptoms such as nasal obstructions and/or rhinorrhea, which may be accompanied by facial fullness or pain, and an impairment of the smell function that worsen after day five, or persist for 10 days to 12 weeks. About 0.5-2% of post-viral acute rhinosinusitis is associated with acute bacterial rhinosinusitis.(2)

Data from the outpatients in Department of ENT (Rhinology-Allergy Division), Dr. Hasan Sadikin Hospital, Bandung, Indonesia in 2011, depicted almost 46% cases of

acute and chronic rhinosinusitis, otherwise in Makassar, about 41.5% rhinologic cases were reported in teaching hospitals, during 2003-2007.(3,4) In the USA, acute rhinosinusitis is one of the ten most common illnesses among workers, thus reducing productivity, and also include in the five most common diseases for which antibiotics are prescribed although most studies show that antibiotics are of little benefit in mild, moderate and uncomplicated cases, while surveys of general practitioners and ENT specialists in Asia show an increase in the use of antibiotics as first line of treatment in cases of moderate (45.9%) and severe (60.3%) acute rhinosinusitis.(1,2,5)

Inflammation in rhinosinusitis generally begins with a viral infection, but in certain circumstances, it can develop into a bacterial infection. It is thought that untreated acute bacterial rhinosinusitis can lead to orbital complications, and severe intracranial infections.(6,7) Several factors can increase the risks of bacterial superinfection in acute rhinosinusitis, including host factors, such as allergy, immune dysfunctions, impaired ciliary functions, and epithelial barrier damage after a viral infection, including from pathogenic factors such as bacterial virulence.(2) Common bacteria thought to be the etiology agents, and *H. influenzae* included as commons pathogens.(8)

Haemophilus influenzae is Gram-negative bacteria, which frequently colonizes the nasopharynx without manifesting symptoms, but can lead to infections through a horizontal spread to the surrounding area.(9) The non-encapsulated or non-typical *H. influenzae* (NTHi) strain is much more frequent in upper respiratory infections than *H. influenzae* encapsulated type b (Hib). These bacteria tend to use several virulence factors in order to survive in the host microenvironment, and like most Gram-negative bacteria, lipopolysaccharide (LPS) is an important virulence factor for *H. influenzae* because it is a major component of the outer membrane that mediates interactions with the host immune system. The LPS in the *H. influenzae* is unique because there are no repeated O-antigen side chains that are usually found in LPS of most Gram-negative bacteria, so they are commonly called lipooligosaccharides (LOS).(9,10) The clinical relevance of LOS structure to the NTHi strain, which is naturally less invasive, is enhancing the ability to induce an inflammatory response. There are variations in the LOS structure among non-typical *H. influenzae* strains, and one that is highly conserved is phosphorylcholine (ChoP).(11) Because the LPS structure of *H. influenzae* does not have a saccharide-bound O-antigen side chain unit, ChoP binds to hexoses in the outer core of LPS, so that ChoP in *H. influenzae* is located on the cell surface. The presence

of ChoP on the cell surface leads to the phase variation of the LOS structure. Phase variation is a random process resulting from reversible gene activity that makes the clonal population of *H. influenzae* appear as heterogeneous phenotypes.(11,12)

The NTHi strain, which colonizes the human upper respiratory tract, has long tandem repeat genes that result in rapid phase variation, and a repeating tetranucleotide region (5'-CAAT-3') in the *lic1A* gene has been identified which plays a role in phase variation of the ChoP epitope in the LOS.(12) The ChoP phase variation allows the *H. influenzae* to make a phenotypic difference in adapting to various environmental conditions. The expression of the ChoP in the LOS will increase the adhesion of the *H. influenzae* to the respiratory tract epithelium, through the interaction with the receptor-platelet activating factor (rPAF).(13) Because of that, the *lic1A* gene is considered to be an important virulence factor, which allows the *H. influenzae* to survive by forming microcolonies, therefore reducing its susceptibility to antimicrobial peptides, and modulating more severe inflammatory processes.(13,14) Bacterial colonization is recognized as the initial stage of the bacterial infection phase of the post-viral acute rhinosinusitis. Unfortunately, there is limited information about the involvement and importance of the NTHi *lic1A* gene, with regards to clinical symptoms associated with acute rhinosinusitis cases.

In the present study, we identified the presence of the NTHi *lic1A* gene, which is known to play an essential role in the colonization of the *H. influenzae* in the mucosa of upper respiratory airway, and its potential correlation with the severity of the disease.

Methods

Study Design and Subjects

Sixty subjects were recruited consecutively in an ENT clinic from a referral hospital. The inclusion criteria covered patients aged >18 years old, who were diagnosed with post-viral acute rhinosinusitis, and had been identified with an onset of two or more symptoms, *i.e.*, nasal obstruction, anterior or posterior mucopurulent secretions, facial pain/pressure, reduction or loss of smell, and symptoms which worsened after five days, or still persisted for more than ten days.(2) The exclusion criteria covered factors such as an intake of antibiotics within ten days before recruitment, a history of chronic rhinosinusitis and immunocompromised, and having dental infections. The enlisted subjects were

asked to participate in this study and signed an informed consent form. The study was reviewed, and approved by The Health Research Ethics Committee, of the Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia (No. 677/UN6.C.10/PN/2017).

Data Collection

The sociodemographic data was recorded, including the patient's age, sex, marital status, education level, history of smoking, and the type and duration of the symptoms. Data regarding the subject's nasal obstruction was measured via a nasal obstruction symptom evaluation (NOSE) scale, and the disease's severity was categorized using a visual analogue scale (VAS).(15,16)

Sampling Procedure

Subjects were given intranasal decongestants and topical anaesthetics while under endoscopic visualization. First, patient was positioned facing the examiner, and the patient's head was gently tilted up around 70°. The swab was inserted into the nostril by tracing the base of the nose medially (nasal septum) and with endoscopic guidance directed towards the middle meatus area, and placing it on the mucous membrane to collect a secrete. Rotate the swab at least 3 times, or up to 5 times if the patient tolerates the procedure well, then carefully withdraw the nasopharyngeal swab and place the swab into the skimmed milk-tryptone-glucose-glycerin (STGG) transport medium (in-house). The samples were transported to the Microbiology laboratory, at the Faculty of Medicine, Universitas Padjadjaran, using an icebox. The transportation occurred within a 4 hours timeframe, and the aliquot was transferred into two new cryovials. A vortex was conducted for 10-20 seconds, and stored at -80°C until use.

PCR Amplification of NTHi *lic1A* Gene

A sample of aliquots which consisted of approximately 200 μ L was used for lysis and DNA extraction procedures. The total DNA extraction used Qiagen blood and a tissue kit

(Qiagen, Hilden, Germany), and was carried out according to the company's recommended procedures. The DNA concentration was calculated using an Infinite Tecan m2000 system (Tecan, Männedorf, Switzerland) after which it was homogenized and diluted to the same concentration across all samples. A total of 1 μ L of the purified DNA was taken and placed in a 100 μ L tube to amplify the DNA using 96 well-thermocycler plates.

The Primer used to amplify the NTHi *lic1A* gene (165 bp) consisted of the forward (F): 5'-GTA GGA TTT GTT AAA ACT TGC TAC AAG CCG-3' and reverse (R): 5'-GGC AAT TCC TCT AAC AGT TTA AAT GCT GCG-3'.

PCR reaction was carried out across 30 cycles, covering 1 minute at 94°C for denaturation, 1 minute at 55°C for the annealing primer, and 1 minute at 72°C for an extension. The final result of the reaction was checked against the band for the electrophoresis process. The amplification bands were analyzed using the Image J software.

Statistical Analysis

The data was validated using a homogeneity test (Leven test), and a normality test (Kolmogorov-Smirnov test). The chi-square test was used to identify the differences in the severity of the disease and the nasal obstruction, based on the presence of the NTHi *lic1A* gene. The data was considered significant if the *p*-value was <0.01, or <0.05. The analysis statistics work was performed using the SPSS software (IBM Corporation, Armonk, NY, USA) version 15.0 for windows.

Results

The 60 patients who were diagnosed with post-viral acute rhinosinusitis, had met the criteria of this study, and had signed the informed consent form. There were 8 secrete samples from osteomeatal complex area of sinonasal patient, with a PCR result which showcased the 5'-CAAT-3' repeat of the NTHi *lic1A* (represented in the Figure 1).

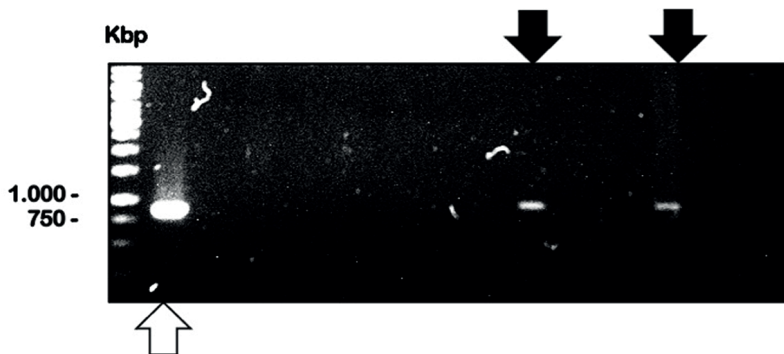


Figure 1. Representative photo of electrophoresis patients sample with positive NTHi *lic1A* gene expression. White arrow indicated positive control sample and a black arrow indicated sample patients with positive NTHi gene expression.

The patients were classified into two groups according to the presence of the *lic1A* genes: eight (13.3%) had the NtHi *lic1A* (+) and 52 (86.7%) did not have the NtHi *lic1A* (-). In this study, 71.7% of the participants were women. The mean age was 32.0 years for the subjects with NtHi *lic1A* (+), and 31.5 years for those without the NtHi *lic1A* (-). Forty-one (68.3%) patients were married, seventeen (28.3%) had completed high school, and twenty-six (48.3%) had a bachelor's or master's degree (Table 1).

Both groups were homogeneous in terms of the education level and monthly income. Although the proportion of those who smoked in the NtHi *lic1A* (+) group was higher (87.5%), there was no significant difference. Regarding the use of drugs all patients with the NtHi *lic1A* (+) (100%) and the majority of those in the NtHi *lic1A* (-) (86.5%) group, took antibiotics over the past one year. There was no significant difference between them.

Nasal Symptoms

Nasal obstruction and nasal discharge are the most common symptoms across both groups. The duration of the symptoms in the NtHi *lic1A* (+) lasted twice as long (mean 28 days vs. 14 days). More than half of the patients had a fever across both groups. A loss of the sense of smell was more common in the NtHi *lic1A* (-) group. More than two-thirds of the patients with NtHi *lic1A* (+) had frontal pain (83.3%), but facial pain/pressure was more frequently associated with patients in the NtHi *lic1A* (-) (70.8%) group, although no

significant differences was seen between these groups, as shown in Table 2.

Disease Severity

We used the VAS scale to measure the severity of the disease. This instrument is widely used to measure the severity of a patient's symptoms. It is clinically relevant, reliable, and easy for patients and healthcare providers to perform.(16) The difference in disease severity based on the NtHi *lic1A* gene's presence is shown in Table 3. Subjects with NtHi *lic1A* (+) were more likely to showcase a severe VAS scale (70%). It is also shown that subjects with NtHi *lic1A* (+) tended to have a much larger VAS scale (mean VAS 8.0 vs. 6.0). Statistical analysis showed a significant difference in the disease's severity between these groups ($p=0.035$).

The Degree of Nasal Obstruction

We used the NOSE scale for measuring the degree of nasal obstruction, which is generally used to assess outcomes of treatments.(15) The NOSE scale is simple, valid, easy to complete, with the potential to assess the severity of complaints experienced by patients during the past months. (17) This studies showed that patients with the NtHi *lic1A* (+) were mostly in the fairly-bad category of for nasal obstruction (62.5%) compare to the NtHi *lic1A* (-) group, which had more moderate nasal obstruction (52.0%). The chi-square analysis showed no significant difference between the groups ($p=0.159$) (Table 4).

Table 1. Sociodemographic characteristics of the study participant.

Demographic Characteristic	NtHi <i>lic1A</i> Gene Positive (n=8)	NtHi <i>lic1A</i> Gene Negative (n=52)	<i>p</i> -value
	n (%)	n (%)	
Age, mean±SD, years	32.0±8.1	31.5±9.3	0.878
Marital status			0.939
Not married	2 (25.0)	15 (28.9)	
Married	6 (75.0)	35 (67.3)	
Divorced	0 (0.0)	1 (1.9)	
Widow/widower	0 (0.0)	1 (1.9)	
Education			0.913
Elementary school	0 (0.0)	3 (5.8)	
Junior high school	0 (0.0)	4 (7.7)	
High school	2 (25.0)	15 (28.9)	
Diploma 3	1 (12.5)	6 (11.5)	
Bachelor degree	4 (50.0)	19 (36.5)	
Magister degree	1 (12.5)	5 (9.6)	
Income, median (IQR), million IDR	6.0 (5.0– 10.0)	7.0 (3.5–10.0)	0.775
Current smoker or passive smoker	7 (87.5)	29 (55.8)	0.088
Antibiotic use within one year	8 (100.0)	45 (86.5)	0.270
Steroid use	2 (25.0)	18 (36.0)	0.543

Table 2. Frequency of symptoms in post-viral acute rhinosinusitis.

Symptoms Characteristic	NTHi <i>lic1A</i> Gene Positive (n=8)	NTHi <i>lic1A</i> Gene Negative (n=52)	<i>p</i> -value
	n (%)	n (%)	
Nasal obstruction	8 (100.0)	52 (100.0)	
Nasal discharge	8 (100.0)	49 (94.2)	0.486
Facial pain/pressure	3 (42.9)	34 (70.8)	0.141
Smell disturbance	4 (57.1)	40 (83.3)	0.106
Fever	5 (71.4)	25 (52.1)	0.337
Frontal pain	6 (85.7)	35 (72.9)	0.468
Postnasal drip	5 (71.4)	38 (79.2)	0.643
Symptoms duration, mean (IQR), days	28.0 (7.0 – 56.0)	14.0 (7.5 – 28.0)	0.691

Discussion

Assessment of the severity of post-viral acute rhinosinusitis symptoms is an important factor in determining the degree of disease and the choice of treatment, in an effort to prevent further complications due to acute bacterial rhinosinusitis (ABRS).(2) From the symptoms point of view, post-viral acute rhinosinusitis are rather nonspecific, however, patients usually present purulent nasal discharge, nasal congestion, facial pain and tenderness, and possible fever. Our study found that nasal discharge and nasal obstruction are the most common symptoms.

One of the factors that are considered relevant to the severity of these sinonasal symptoms is the influence of predominant bacteria, including aerobic bacteria that are usually found as a causative bacteria such as *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis*.(18,19) One of those bacteria, *H. influenzae*, shows the ability to perform phase variations CHO-P-LOS structure that encoded by the *lic1A* gene (12), which in turn increases the adhesion of *H. influenzae* to the epithelium of the respiratory tract through interaction with the receptor-platelet activating factor (rPAF), decreased antibody binding to LPS, thereby avoiding antibody-dependent complement activity.(13,20) In this study, the NTHi *lic1A* gene was analyzed in clinical

isolates obtained from the meatus media of post-viral acute rhinosinusitis patients, as a representation of the variation of the ChoP phase in LOS that may have appeared during the translocation of *H. influenzae* in the nasopharynx to the sinonasal space. The identification of the NTHi *lic1A* gene in this study provides preliminary evidence of *H. influenzae* colonization in the sinonasal mucosa of post-viral acute rhinosinusitis patients.

In relation to the severity of the disease, this study showed that subjects with a positive NTHi *lic1A* gene tend to have a much more severe illness. Previous studies have indicated that during the nasopharyngeal carrier stage, the phenotype ChoP (+) increases with the longer duration of the *H. influenzae* colonization.(21) Consistent with our study, a longer duration of symptoms occurred in patients with the NTHi *lic1A* (+). However, it is not clear why post-viral acute rhinosinusitis patients with the NTHi *lic1A* (+) tend to have more severe complaints, and a longer symptom duration. This could be attributed to the increased adherence of the *H. influenzae* ChoP (+) variant into the host cells, and may lead to increased bacterial resistance to the host's antimicrobial peptides and modulate a much more severe inflammatory response.(13,14)

This studies also showed that patients with the NTHi *lic1A* (+) were mostly in the fairly-bad category of nasal obstruction, nonetheless, statistic analysis showed no

Table 3. The severity of disease and the presence of NTHi *lic1A* gene.

VAS Scale	NTHi <i>lic1A</i> gene Positive (n=8)	NTHi <i>lic1A</i> gene Negative (n=52)	(95% CI)	<i>p</i> -value *
	n (%)	n (%)		
Mild	0 (0.0)	8 (15.4)	Ref	
Moderate	2 (25.0)	29 (55.8)	(1.04-2.6)	0.035
Severe	6 (75.0)	15 (28.8)		
Mean±SD	8.1±1.4	6.0±2.5		

**p*-value was calculated using the Chi-squared test for categorical variables.

Tabel 4. The degree of nasal obstruction and the presence of NTHi *lic1A* gene.

NOSE Scale	NTHi <i>lic1A</i> gene Positive (n=8)	NTHi <i>lic1A</i> gene Negative (n=52)	<i>p-value</i> *
	n (%)	n (%)	
Mild	2 (25.0)	4 (7.7)	0.159
Moderate	1 (12.5)	26 (52.0)	
Fairly bad	5 (62.5)	21 (40.4)	
Severe	0 (0.0)	1 (1.9)	

**p-value* was calculated using the Chi-squared test for categorical variables.

significant difference between the groups. However, nasal obstruction itself remained a diagnostic challenge for the clinicians, considering the fact that nasal obstruction is much more of a subjective complaint. It is difficult to predict, since there are often inconsistencies between nasal obstruction felt by the patient, and the objective assessment of the nasal congestion/patency using various instruments.(22)

Current evidence suggest that the ChoP epitope modulation in LOS *H. influenzae* is likely to be encoded by several other genes, such as *galU* and *csrA*.(23,24) Although we did not examine those genes, and we also did not assess the activity and levels of *lic1A* mRNA, as well as the ChoP phase variation during *H. influenzae* colonization in the sinonasal mucosa of post-viral acute rhinosinusitis patients which could confirm the transcription performance of the *lic1A* gene during ChoP protein biosynthesis in the LOS structure, however, our studies indicates the examination of NTHi *lic1A* genes can be beneficial in determining prognosis of post-viral acute rhinosinusitis patients and could potentially be a good marker for assessing the severity of acute rhinosinusitis.

Conclusion

Knowledge regarding the correlation between the degree and severity of post-viral acute rhinosinusitis and the presence of *H. influenzae* colonization in the sinonasal mucosa can be used as a reference for the importance of managing microaerobic conditions in the sinonasal cavity, especially those related with *H. influenzae* bacteria in maintaining patient susceptibility to complications of bacterial infection. The presence of the NTHi *lic1A* gene is significantly associated with disease severity and the duration of the symptoms. Thus, the NTHi *lic1A* gene could potentially be a good marker for assessing the severity of post-viral acute rhinosinusitis cases in the future.

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Authors Contribution

IM performed study design, data gathering and analysis, interpretation of data, and preparation of article draft. RL were involved in data gathering and analysis, interpretation of data, and critical review. NS performed the measurements and statistical analysis. SS and TM supervised, critical review, and approval of final version of manuscript. All authors discussed the results and commented on the manuscript.

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