The Relationship of iTILs (Intratumoral Infiltrating Lymphocytes) and sTILs (Stromal Infiltrating Lymphocytes) with SOX2 (Sex-Determining Region Y (SRY)-Box2) Expression in Nasopharyngeal Carcinoma

Asyrafun Nisa Adelaidey[a*], Lidya Imelda Laksmi[b], Causa Trisna Mariedina[b], M. Nadjib D Lubis[b], Tengku Kemala Intan[b], Soekimin[b]

[a]Master in clinical medicine, Anatomical Pathology, Faculty of Medicine, University of North Sumatra, University of North Sumatra Hospital
[b]Department of Anatomical Pathology, Faculty of Medicine, University of North Sumatra, Hospital, University of North Sumatra
email: 21dec84@gmail.com

Abstract

Background: Nasopharyngeal carcinoma (NPC) is a type of malignant head and neck tumor originating from the epithelial lining of the nasopharynx which is commonly found in Asian countries, including Indonesia. SOX2 is part of SOX. The SOX gene family first emerged with the discovery of the mammalian testicular determinant, Sry. SOX2 expression in NPC is promoted by transcription factors such as activation protein 2 (AP-2), homeobox protein 1 (Prox1) and Pax6. TILs can be used systematically to evaluate a patient's anti-cancer immunological function that can be a valuable prognostic factor.
Research Objectives: To determine the relationship between the degree of iTILs (intratumoral infiltrating lymphocytes) and sTILs (stromal infiltrating lymphocytes) with the immunohistochemical expression of SOX2 (sex-determining region Y (SRY)-box 2) in the histopathology of nasopharyngeal carcinoma.
Materials and Methods: 43 samples in the form of a histopathological slide from biopsy/operative tissue diagnosed with nasopharyngeal carcinoma were collected. Stromal and intratumoral TILs were evaluated by the researcher and two anatomic pathologists, then immunohistochemical staining of SOX2 was performed and the expression was assessed. Spearman correlation test was performed to analyse between variables.
Results: Based on the Spearman's correlation test, there was no significant relationship between the degree of iTILs and SOX2 expression (p= 0.892) and there was a significant relationship between the degree of sTILs and SOX2 expression (p= 0.003).
Conclusion: This study shows no significant relationship between the degree of iTILs and SOX2 expression, but there was a significant relationship between the degree of sTILs and SOX2 expression.

Keywords : iTILs, sTILs, Sex determining region Y box 2, carcinoma, nasopharingx.

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a type of malignant head and neck tumor originating from the epithelial lining of the nasopharynx which is commonly found in Asian countries, including Indonesia. The postero-lateral nasopharynx or pharyngeal recess (Rossenmüller fossa) are the most common site of NPC.[1] Microscopically and ultrastructurally, squamous differentiation of NPC includes non-keratinizing, keratinizing, and basaloid squamous carcinoma.[2]

Based on GLOBOCAN (Global Burden of Cancer) 2020, the incidence of NPC is constantly rising. There are 133,354 cases of NPC per year with a mortality rate of 80,008 cases per year.[3] According to the

latest statistics, about 80% of NPC incidency is in Asia, especially in Southeast Asia with 65,866 new cases found and 36,453 deaths. In Indonesia, NPC is dominated by men and ranks 4th of cancer in men after lung cancer, colon cancer and liver cancer, with an incidence rate of 15,427 cases.[3,4,5]

However, about 5-15% of patients still have local recurrence and 15-30% of patients have distant metastases.[6] For this reason, the management of NPC in recent years has focused on identifying the cellular and molecular mechanisms of treatment resistance and metastases that are the main targets in the discovery of treatments and the design of new drugs.[4] Most patients with NPC are sensitive to both radiotherapy and chemotherapy. However, treatment failure remains high due to the development of local recurrence and distant metastases.[7,8]

SOX2(sex-determining region Y (SRY)-box 2) is one of the molecular biology target therapy being developed. Based on gene profiling, SOX2 is a marker for cancer stem cells (CSC) in various organs including NPC. The majority of deaths in NPC are associated with tumor metastases compared to the primary tumor itself.[9] Research by Wang et al. in NPC patients showed that strong SOX2 expression was significantly associated with poorer distant metastasis-free survival (DMFS) compared to weak SOX2 expression.[10] Luo, et.al also showed that NPCs with strong SOX2 expression have a poorer prognosis than those that are underexpressed.[7]

SOX2 expression in NPC is promoted by transcription factors such as activation protein 2 (AP-2), homeobox protein 1 (Prox1) and Pax6. E2f3a cooperates with pRb family member p107 to suppress SOX2 expression, whereas E2f3b activates SOX2 expression by recruiting RNA polymerase II to its promoter. Cyclin-dependent kinase inhibitor p21 has also been found to directly bind to SOX2 enhancers and suppress SOX2 expression in NPC.[11,12] SOX2 also plays a role in identifying distant metastases from NPC tumors because of its rapid spread and high invasion. However, in some literature, the role of SOX2 in tumorigenesis is still controversial

At present, the relationship between a high number of Tumor Infiltrating Lymphocytes (TILs) and a good prognosis has been investigated in various types of cancer.[13,14] However, the prognostic value of TILs in NPC is not adequate, although several studies have reported an association between lymphocytes, overall survival and overall or disease-free survival in NPC.[15] TILs can be used systematically to evaluate a patient's anti-cancer immunological function, so they can be a valuable prognostic factor.[16] TILs can be used for immunotherapy, including CTLA-4 and PD-1/PD-L1 antibodies. Therefore, further understanding of TILs is very important. Ex vivo culture of NPC biopsy specimens showed TILs can be used as T-cell-based immunotherapy in NPC patients. Although TILs have shown some therapeutic effect, the prognostic effect is still being ignored.[16,17]

SOX2 (sex-determining region Y (SRY)-box 2) is a regulator of various types of stem cells, especially embryonic stem cells (ESCs) and neural progenitor cells (NPCs).[18] SOX2-specific-T lymphocytes stimulated by CD 154-activated B cells in combination with immune checkpoint inhibitors have a strong tumoricidal effect. Examination of SOX2 expression with CD8+ TILs levels can help determine the prognosis.[19,20]

Currently, research on the relationship of SOX2 expression and TILs in NPC has not been carried out. According to Lee et al. in small cell lung carcinoma showed that the combination of high expression of SOX2 and CD8+ TILs provided a good prognostic factor with significantly longer overall survival (OS) and progression free survival (PFS).[21] Therefore, the researcher wanted to examine the relationship between SOX2 expression and TILs in NPC in Medan.

MATERIALS AND METHODS

There were 43 samples in the form of histopathological slides from biopsy/operative tissue diagnosed with nasopharyngeal carcinoma that were collected in Adam Malik General Hospital Medan, after obtaining

approval from the Health Research Ethics Committee, Faculty of Medicine, University of North Sumatra.

Slides were reviewed, then stromal and intratumoral TILs were assessed by the researcher and two anatomic pathologists, then SOX2 immunohistochemical staining was performed Anti-SOX-2 primary monoclonal mouse antibody (Bioassay), 1:50 dilution).and the expression was assessed. Spearman correlation's test is used to analyse correlation between variables.

RESULTS

In this study, 43 samples of nasopharyngeal carcinoma that met the inclusion criteria at Adam Malik General Hospital Medan were collected, which aims to analyze the relationship between the degree of iTILs (intratumoral infiltrating lymphocytes) and sTILs (stromal infiltrating lymphocytes) with immunohistochemical expression of SOX2 (sex-determining region Y (SRY)-box 2) on histopathology of nasopharyngeal carcinoma.

Table 1. Frequency distribution of nasopharyngeal carcinoma patients' characteristics, degree of intratumoral TILs, stromal TILs and SOX2 expression.

	Amount	Percentage
Age		
• 20 years	1	2.3
• 21 – 40 years	6	14
• 41 – 60 years	32	74.4
• >61 years	4	9.3
Gender		
• Man	25	58.1
• Woman	18	41.9
Subtype		
• NKSCC		
Undifferentiated	31	72.1
Differentiated	5	11.6
• KSCC	7	16.3
• Basaloid squamous cell carcinoma	0	0
Degree of iTILs		
• High	17	39.5
• Low	26	60.5
Degree of sTILs		
• High	19	44.2
• Low	24	55.8
Expression of SOX2		
• Weak	23	53.5
• Moderate	8	18.6
• Strong	12	27.9

In Table 1, the characteristics of nasopharyngeal carcinoma patients are assessed by age, which in this study was divided into 4 age categories with the number of patients < 20 years old in 1 case (2.3%), age 21-40 years with a total of 6 cases (14%), 41-60 years old in 32 cases (74.4%) and aged > 60 years in 4 cases (9.3%). Based on gender, patients with nasopharyngeal carcinoma were found in mostly male patients with a total of 25 cases (58.1%), while female patients were found in 18 cases (41.9%). Based on histopathological type, patients with NKSCC was the most common cases, which was found in 36 cases (83.7%), KSCC was only found in 7 cases (16.3%) and no cases of Basaloid squamous cell carcinoma were found in this study. In patients with NKSCC, it is known that there are more undifferentiated subtypes, which was found in 31 cases (72.1%) compared to differentiated, which were found in 5 cases (11.6%). The assessment of TILs in this study was divided into intratumoral TILs and stromal TILs. High intratumoral TILs were found in 17 cases (39.5%) and low in 26 cases (60.5%). Meanwhile, high stromal TILs were found in 19 cases (44.2%) and low stromal TILs in 24 cases (55.8%).The highest degree of SOX2 expression in this study was found in 23 cases (53.5%), strong in 12 cases (27.9%) and moderate in 8 cases (18.6%).

Table 2. Distribution of iTILs degree based on histopathological subtype of nasopharyngeal carcinoma.

Degrees of iTILs	Diagnosis								Total
	KSCC		NKSCC Undifferentiated		NKSCC differentiated		SCC Basaloids		
	n	%	n	%	n	%	n	%	
High	0	0	15	48.4	2	40	0	0	17
Low	7	100	16	51.6	3	60	0	0	26
Total	7	100	31	100	5	100	0	0	43

In Table 2, it is known that high iTILs were not found in histopathological subtypes of KSCC and Basaloid SCC, but were found in NKSCC, both undifferentiated in 15 (48.4%) cases and differentiated in 2 (40%) cases. Meanwhile, low iTILs were not found in the Basaloid SCC, but were found in the undifferentiated and differentiated KSCC and NKSCC subtypes of 7 (100%), 16 (51.6%) and 3 (60%) cases respectively.

The frequency distribution of sTILs by histopathological subtype of nasopharyngeal carcinoma is presented in the table (Table 3).

Table 3. Distribution of sTILs grade by histopathological subtype of nasopharyngeal carcinoma

Degrees of sTILs	Diagnosis								Total
	KSCC		NKSCC Undifferentiated		NKSCC differentiated		SCC Basaloids		
	n	%	n	%	n	%	n	%	
High	5	71.4	13	41.9	1	20	0	0	19
Low	2	28.6	18	58.1	4	80	0	0	24
Total	7	100	31	100	5	100	0	0	43

In Table 3, it is known that high sTILs were found in the undifferentiated NKSCC in 13 (41.9%) cases, followed by the KSCC in 5 (71.4%) cases and differentiated NKSCC in 1 (20%) case, but not in the Basaloid SCC type. Meanwhile, low sTILs were found in 18 (58.1%) undifferentiated NKSCC subtypes, followed by 4 (80%) differentiated NKSCC and 2 (28.6%) KSCC. Basaloid type KSCC was not found in this study.

In this study, each sample was assessed for the degree of iTILs based on the expression of SOX2 in nasopharyngeal carcinoma. Furthermore, the correlation test of iTILs degree and SOX2 expression was carried out as presented in the table (Tables 4.4 and 4.5).

Table 4. Distribution of iTILs grade based on SOX2 expression in nasopharyngeal carcinoma.

Degree of iTILs	SOX2 Expression						Total
	Weak		Moderate		Strong		
	n	%	n	%	n	%	
High	9	37.5	5	62.5	3	27.3	17
Low	15	62.5	3	37.5	8	72.7	26
Total	24	100	8	100	11	100	43

In Table 4, degree of high iTILs and low SOX2 epression were found in 9 samples (37.5%), followed by 5 samples (62.5%) who were moderate and 3 samples (27.3%) were strong. Low iTILs and low SOX2 expression were found in 15 samples (62.5%), followed by strong SOX2 expression in 8 samples (72.7%), and moderate SOX2 epression in 3 samples (37.5%).

Table 5. Correlation of iTILs and expression of SOX2 in nasopharyngeal carcinoma

Variable	Expression of SOX2		
	n	r	P
iTILs	43	0.021	0.892

***Spearman Correlation test**

In Table 5, the results of the correlation test shows that there is no significant relationship between the degree of iTILs with SOX2 expression in nasopharyngeal carcinoma through Spearman correlation test, where the value of p=0.892 (p>0.005).

In this study, sTILs were assessed in each sample based on SOX2 expression. Furthermore, correlation test was used to assess the degree of sTILs and SOX2 expression, as presented in the following table (table 6 and 7).

Table 6. Distribution degree of sTILs based on SOX2 expression in nasopharyngeal carcinoma.

Degree of sTILs	Expression of SOX2						Total
	Weak		Moderate		Strong		
	n	%	n	%	n	%	
High	7	29.2	2	25	10	90.9	19
Low	17	70.8	6	75	1	9.1	24
Total	24	100	8	100	11	100	43

In Table 6, degree of high sTILs were found in 7 samples (29.2%) with weak SOX2 epression, followed by 2 samples (25%) who were moderate and 10 samples (90.9%) were strong. While the degree of low sTILs were found in 17 samples (70.8%) with weak SOX2 expression, followed by 6 samples (75%) who were moderate and 1 sample (9.1%) was strong.

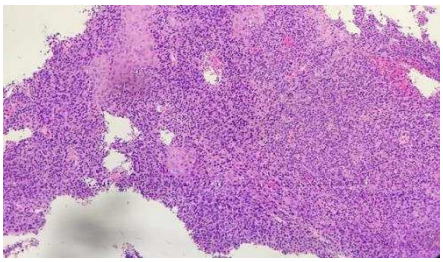
Table 7. Correlation test of sTILs and SOX2 expression in nasopharyngeal carcinoma

Variable	Expression of SOX2		
	n	r	P
sTILs	43	0.448	0.003

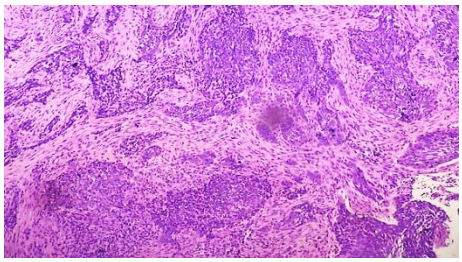
***Spearman test**

In table 7, the results of the correlation test show that there is a significant relationship between the

degree of sTILs and SOX2 expression nasopharyngeal carcinoma through Spearman correlation test, where the value of p=0.003 (p<0.005).



i-Tils: low, s-Tils: high (HE 100x)



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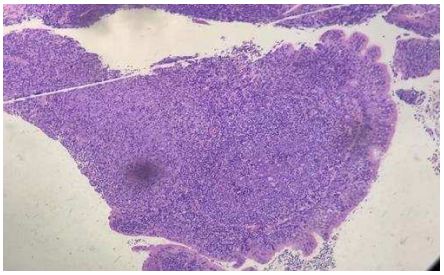
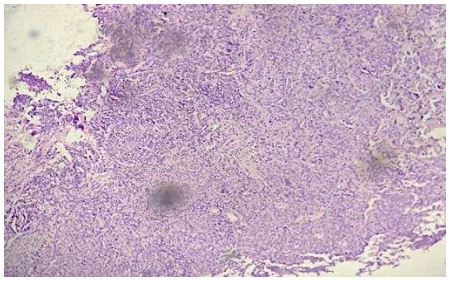


i-Tils: high , s-Tils: high (HE 100x)



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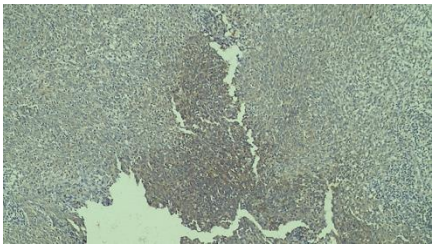
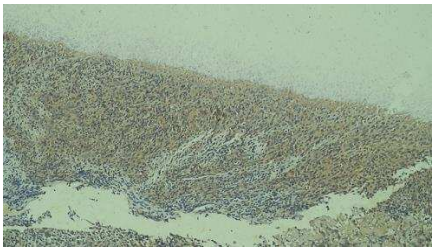
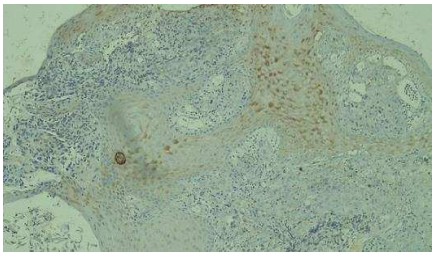


Strong SOX2 expression



Moderate SOX2 expression



Weak SOX2 expression

DISCUSSION

In this study, patients with nasopharyngeal carcinoma were predominantly found in the 41-60 years age group with a total of 74.4% of cases. The youngest case is found in 20 years old and the oldest is 71 years old. The results of this study are in line with the research conducted by Farhat et al., at H. Adam Malik Hospital in 2015 – 2016, where the highest incidence of NPC patients was 41-60 years old (57.1%) and this is also in line with the research of Kuswandi et al., at RSUD Dr. Abdul Moeloek in 2016 – 2019 in Lampung, where the most incidence of NPC was found in the 46-55 year age group, namely 28.6%.[22] This is in accordance with the statement that NPC incidence begins to increase after the age of 30 years with a peak incidence of 40- 60 years and then decreases. This condition is related to the function of DNA repair mechanisms (DNA repair) and the immune system that are decreasing when experiencing mutations after the age of 40 years. Previous research stated that there are 3 main etiologies of NPC, namely genetic predisposition, prolonged exposure to chemical carcinogens, consumption of salted fish or fermented food from a young age, and EBV infection.[23]

More than half NPC was found in men. This is in line with research conducted by Suta et al., at Sanglah Hospital in 2019, where the highest incidence of NPC was found in male and this could be due to lifestyle factors such as smoking and exposure to carcinogenic substances.[24] Based on histopathological type, NKSCC subtypes were present in most cases, namely 36 (83.7%) cases and undifferentiated subtype in 31 (72.1%) cases and no cases of basaloid squamous cell carcinoma were found in this study. Unsal DO et al., said that cases of basaloid squamous cell carcinoma are very rare, only 6 out of 100 million patients in the United States.[25] This research is in line with Kuswandi et al., at Dr. Hospital. Abdul Moeloek in 2016 – 2019 in Lampung with the highest number in NKSCC subtype undifferentiated as much as 71.4%. Pathogenically, undifferentiated NKSCC is closely related to environmental and lifestyle factors and often occurs in the Rosenmuller fossa which indicates that carcinogens enter through the respiratory tract, either from air from the atmosphere or folate components from food. In addition, consumption of salted fish is one of the risk factors that can cause undifferentiated NKSCC because the folate content in the form of nitrosamines is a potential oncogene.[26]

Lymphocyte infiltration to tumor microenvironment (TME) generally considered to represent host immunity against tumors. To date, most studies have evaluated the relevance of infiltrating T cells in TME. TILs are immune cells that are triggered by a host immune response to tumors, including T cells (CD4+/Th helper T lymphocytes, CD8+/CTL cytotoxic T lymphocytes, and FOXP3+/Treg regulatory T cells), macrophages, dendritic cells, and mast cells. The most dominant and consistent characteristic of NPC is the presence of a very abundant lymphocyte infiltrate and is associated with a high rate of local invasion and locoregional lymphatic metastases.[27]

TILs in NPC is associated with prognostic factors and the development of immunotherapy. High TILs levels are associated with better outcomes and survival rates and TILs has been said to exhibit effective anti-tumor immune response, induce immune response, delayed tumor development, and enhance the immune-cancer microenvironment.[27]

The assessment of the degree of TILs in this study was based on intratumoral TILs and stromal TILs. iTILs was mostly found in low grade which was distributed mostly in undifferentiated NKSCC as many as 51.6% cases and in KSCC was found in 100% of cases in low grade. This research is in line with Almangush et al., where iTILs in KSCC were most commonly found in low grades of 50%, undifferentiated NKSCCs were mostly found in high grades of 76%, and differentiated NKSCCs at high grades of 13.3%. Low iTILs in KSCC are associated with poor overall survival (OS) and disease specific survival (DSS).[28]

Whereas sTILs on KSCC is mostly found in high degree, in as many as 71.4% of cases and differentiated NKSCC is modtly found in low degrees as many as 58.1% of cases. Almangush et al., in Finland, showed that iTILs was mostly obtained in high degree (65.2%) and the highest degree of sTILs was found to be high (73%), and there was a significant relationship between EBV and TILs, where tumors with

positive EBV had high stromal and intratumoral TILs. Low intratumoral TILs are associated with poor OS.[28] There are not many studies linking TILs and NPC subtypes by hematoxylin and eosin (H&E) staining. One of the studies, including Tambunan et al., found no significant relationship between TILs and NPC subtypes. However, high TILs were mostly found in undifferentiated NKSCC.[29] TILs is not specific for a particular tumor cell, but can be used as a prognostic factor in tumor type.[29,30]

This study focuses on the relationship of iTILs and sTILs degree with SOX2 in NPC patients. It appears that the low iTILs is mostly found in weak SOX2 expression. However, after the statistical correlation test was carried out with the Spearman correlation test, there was no significant relationship between the degree of iTILs and SOX2 in NPC ($p<0.005$). Although the correlation was not significant, there is a corresponding tendency, where the lower the iTILs is, the weaker SOX2 is expressed and the poorer the prognosis become. Research conducted by Lee et al.,in 2021 was stated that small lung cell carcinoma does not distinguish between iTILs and sTILs, and the concept of immunotherapy is based on the tumoricidal effect of CD8+ T cells, expression of SOX2 and CD8+. A high level indicates a good prognostic factor.[31]

Based on Table 6 high sTILs is mostly found in strong SOX2 epression and low sTILs is mostly found on weak SOX2 expressions. After statistical correlation and Spearman correlation test were conducted, there was a significant relationship between the degree of sTILs and SOX2 in NPC where the value of p is 0.003 ($p <0.005$). It shows a unidirectional relationship between the degree of sTILs and SOX2 expression. So far, we have not found any research between degree of sTILs and SOX2 expression. Suarez-Sanchez et al. assess the relationship of CD20+ B Lymphocytes expression on oral squamous cell carcinoma, where CD20+ TIL is inversely correlated with the expression of NANOG/SOX2. This shows that cancer stem cells contribute to immunity effacion in the evasion of the immune system and contribute to oral squamous cell carcinoma. CD20 and FOXP3 ratio in the stroma was significantly associated with tumor recurrency. It seems contradictory results due to subsite TILs heterogeneity affects the relationship between the two.[32]

CONCLUSION

This study shows no significant relationship between the degree of iTILs and SOX2 expression, but there was a significant relationship between the degree of sTILs and SOX2 expression.

COMPETING INTEREST

The author has no financial interests that are relevant to the products or company described in this article.

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ETHICAL APPROVAL

The Health Research Ethical Commitee, University of North Sumatra, Medan, Indonesia approved this study.

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AUTHORS DETAILS

First Author – dr. Asyrafun Nisa Adelaidey, resident of Anatomical Pathology Departement, Faculty of Medicine, University of North Sumatra, Medan, Indonesia, id email: 21dec84@gmail.com

Second Author – Dr. dr. Lidya Imelda Laksmi, M.Ked(PA), Sp.PA(K), Lectures and Staff Anatomical Pathology Department, Faculty of Medicine, University of North Sumatra, Medan, Indonesia

Third Author – dr. Causa Trisna Mariedina, M.Ked(PA), Sp.PA, Lectures and Staff Anatomical Pathology Department, Faculty of Medicine, University of North Sumatra, Medan, Indonesia

Fourth Author – Prof. dr. M, Nadjib D Lubis, Sp.PA (K), Lectures and Staff Anatomical Pathology Department, Faculty of Medicine, University of North Sumatra, Medan, Indonesia

Fifth Author – dr. Tengku Kemala Intan, M.Pd, M.Biomed, Lectures and Staff Anatomical Pathology Department, Faculty of Medicine, University of North Sumatra, Medan, Indonesia
Fifth Author – dr. Soekimin, Sp.PA(K), Lectures and Staff Anatomical Pathology Department, Faculty of Medicine, University of North Sumatra, Medan, Indonesia

Corresponding Author –Asyrafun Nisa Adelaidey, resident of Anatomical Pathology Department, Faculty of Medicine, University of North Sumatra, Jl. Universitas. No. 1 gedung Abdul Hakim, Medan, Indonesia, E-mail ID: 21dec84@gmail.com