

Correlation of N-Cadherin and MMP-9 Expression with Regional Nodal Metastasis in Laryngeal Squamous Cell Carcinoma

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ABSTRACT

Background: In laryngeal carcinoma, regional nodal metastasis serves as a significant prognostic factor. A special biomarker is needed to predict the status of nodal metastases. N-cadherin, a binding protein, aids in cell migration and enabling tumor cells to spread to new locations. The involvement of matrix metalloproteinase-9 (MMP-9) in metastasis includes fostering the dissemination of tumor cells from the primary tumor and enhancing tumor cell motility. **Objective:** This study seeks to explore the distinctions and relationships in N-cadherin and MMP-9 expression among patients with laryngeal squamous cell carcinoma at various N stages. **Methods:** Employing a cross-sectional approach, this study conducted an analytic observational investigation on formalin-fixed paraffin-embedded patients whose histopathological diagnosis is laryngeal squamous cell carcinoma in the Pathology Laboratory of Dr. Soetomo Regional Public Hospital in 2018–2021. The samples were divided into N stages based on radiological imaging from the CT scan. Immunohistochemistry examinations were performed using N-cadherin and MMP-9 antibodies and scored using the immunoreactive score (IRS), based on percentage and intensity. The differences in expression and correlation between N-cadherin and MMP-9 were analyzed using statistical tests. **Results:** Statistical insignificance was observed in N-cadherin expression at various N stages ($p = 0.099$). There were significant differences in MMP-9 expressions at various N stages ($p = 0.0006338$). There was no correlation between N-cadherin and MMP-9 expression at various N stages in laryngeal squamous cell carcinoma ($p = 0.0638$, $r_s = 0.27$). **Conclusion:** In laryngeal squamous cell carcinoma, MMP-9 serves as a predictor for lymph node metastasis, which, if present, deteriorates the patient's prognosis.

Keywords: Laryngeal cancer, N-cadherin, MMP-9, N stages, Metastasis.

INTRODUCTION

With a mortality rate exceeding 50%, laryngeal carcinoma ranks as the second most prevalent malignancy of the respiratory tract.^{1,2} Based on histopathological features, most laryngeal carcinomas originate from the surface epithelium, and about 90% are conventional squamous cell carcinoma types.³ In laryngeal carcinoma, invasion and metastasis stand out as the most critical prognostic factors.⁴ Laryngeal carcinoma most often metastasizes to regional lymph nodes and lungs [3]. Nodal status has a very significant effect on determining the cure rate compared to the status of the primary tumor.⁵ The prognosis for laryngeal carcinoma worsens when regional lymph nodes are involved.⁴ Total laryngectomy surgery is performed when laryngeal cancer fails to respond to conservative laryngectomy surgery, chemotherapy, radiotherapy, or when the cancer is at an advanced stage (T3, T4). This situation causes a delay in establishing the diagnosis of nodal metastatic status.⁶

Loss of cell-cell adhesion, degradation of the extracellular matrix, and angiogenesis—new blood vessel formation—are hallmarks of cancer metastasis, facilitating tumor growth by supplying essential nutrients.⁷ N-cadherin, an adhesion protein from the cadherin family, aids in cell migration as a whole through a variety of routes, enabling tumor cells to spread to new locations.⁸ The process of epithelial-to-mesenchymal transition (EMT) entails epithelial cells undergoing

a complex transformation where they shed their characteristic traits and acquire the migratory capacity of mesenchymal cells.⁸⁻¹² Unlike E-cadherin, which exhibits a different pattern of expression, N-cadherin is typically found in neuroectodermal and mesodermal cells like nerve cells and cardiac muscle. N-cadherin has a significant involvement in several processes, including adhesion between mesenchymal cells (tends to be unstable and dynamic), differentiation, embryogenesis, migration, and invasion. Tumor progression is linked with aberrant N-cadherin expression in certain epithelial cell tumors. The crucial of N-cadherin makes it an important target for therapy.¹¹

The extracellular matrix is broken down by the matrix metalloproteinase (MMP) protein, which facilitates tumor cell migration. By inducing the escape of tumor cells from their main tumor, enhancing tumor cell motility, and enabling tumor cells to endure at their metastatic sites, MMP-9 contributes to metastasis.⁸

This study aims to analyze the expressions of N-cadherin and MMP-9 in various metastatic (N) states in regional lymph node nodules in the neck based on CT scan imaging in laryngeal squamous cell carcinoma. These findings will help to estimate the prognosis and determined the aggressiveness of treatment in patients.

MATERIAL AND METHODS

This study adopts a cross-sectional approach as part of its analytical observational design. The

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sample of this study is formalin fixed paraffin embedded blocks from biopsy and surgical material that had been diagnosed as laryngeal squamous cell carcinoma in the Anatomical Pathology Laboratory of Dr. Soetomo General Academic Hospital between 2018 and 2021. Data on demographics, comprising age and gender, radiological assessments including TNM staging and lesion sites, and pathology reports, were gathered from the patient's medical file. The total samples used in this study were 48 samples. The N stage was assessed radiologically by CT scan imaging based on the criteria of the WHO Classification of Tumors of the Head and Neck 2017 and the 7th American Joint Committee on Cancer (AJCC) 2020. The samples were divided into 4 groups, namely N0 = 8 samples, N1= 13 samples, N2= 26 samples, and N3= 1 sample. Due to the limitations of the N3 sample, the N3 sample was merged with N2 in statistical tests.

A representative slide of the tumor and its corresponding paraffin block were chosen for each laryngeal squamous cell carcinoma case. The primary antibodies used for N-cadherin are rabbit polyclonal antibody Anti-N-Cadherin, ab18203 (abcam®, Cambridge – UK) with dilution 1:1.500; while MMP-9 used mouse monoclonal antibody MMP-9 (2C3): sc-21733 (Santa Cruz Biotechnology, Europe) with dilution 1:100.

Immunohistochemical expression with the antibodies N-cadherin and MMP-9 were observed in each group using the Olympus CX41RF binocular microscope. N-cadherin is expressed as positive by membrane and cytoplasm of tumor cells becoming brown colored. MMP-9 is expressed as positive by cytoplasm of tumor cells becoming brown colored. The expressions of antibodies are scored using Immunoreactive Score (IRS) by counting the staining percentage and intensity. For the percentage of tumor cells (A), the scores are 0 (none was positive), 1 (positive cells < 10%), 2 (10–50%), 3 (51–80%), and 4 (> 80%). Intensity scores (B) spanned from negative to strong, defined as follows: 0 denoting negative, 1 denoting weak, 2 denoting moderate, and 3 denoting strong. The total immunoreactive score is derived by multiplying the percentage and intensity scores for each specimen, with values spanning from 0 to 12. Categorization of immunohistochemical scores included four groups: negative (0-1), weak (2-3), moderate (4-8), and strong (9-12).^{13,14} In a double-blind fashion, two pathologists conducted the assessment.

Analysis was performed using EZR software. Kruskal-Wallis testing was employed for expression comparison, and correlation analysis was carried out using the Spearman correlation test. A p-value < 0.05 was considered indicative of statistical significance.

RESULTS

The sample population included a total of 48 laryngeal squamous cell carcinoma patients in Dr Soetomo General Academic Hospital, Surabaya Indonesia from 2018 to 2021 (Table 1). The data obtained in this study showed a sex distribution in which male patients (n = 45) were more common than female patients (n = 3). Patients in this group had a mean age of 59.5 years, with ages spanning from 27 to 84 years. The tumor is mostly located in the glottis (32.67%). By nodal status, most patients in this study were in the N2 stage (54%).

Examination of N-cadherin expression through the Kruskal Wallis test demonstrated no significant differences among the distinct N stages in laryngeal squamous cell carcinoma, registering a p-value of 0.099 (p < 0.05) (Table 2). The staining of N-cadherin based on the IRS score is presented in Figure 1.

The Kruskal-Wallis test revealed a significant discrepancy in MMP-9 expression across different N stages in laryngeal squamous cell carcinoma, with a p-value of 0.0006338 (p < 0.05) (Table 3). MMP-9 staining, classified by IRS score, is depicted in Figure 2.

The correlation between N-cadherin and MMP-9 expression was assessed using Spearman's correlation test. The analysis demonstrated

Table 1. Patient's distribution data.

Variable	N
Sex (n = 48)	
– Male	45 (94%)
– Female	3 (6%)
Age (n = 48)	
– 21-30 years	1 (2%)
– 31-40 years	2 (4.2%)
– 41-50 years	5 (10.4%)
– 51-60 years	18 (37.5%)
– 61-70 years	15 (31.25%)
– 71-80 years	6 (12.5%)
– > 80 years	1 (2%)
Location (n = 48)	
– Supraglottis	3 (6%)
– Glottis	32(67%)
– Subglottis	6 (12%)
– Transglottis	7 (15%)
Nodal metastasis by CT scan (n = 48)	
– N0	8 (17%)
– N1	13 (27%)
– N2	26 (54%)
– N3	1 (2%)

Table 2. N-cadherin distribution at various N stages of laryngeal squamous cell carcinoma.

N-cadherin expression	Category	N0	N1	N2	p-value
IRS score	Negative (0-1)	1 (12.5%)	2 (15.38%)	0 (0%)	p=0.099
	Weak (2-3)	2 (25%)	5 (38.46%)	9 (33.33%)	
	Moderate (4-8)	5 (62.5%)	6 (46.15%)	17 (62.96%)	
	Strong (9-12)	0 (0%)	0 (0%)	1 (3.70%)	

Table 3. MMP-9 distribution at various N stages of laryngeal squamous cell carcinoma.

MMP-9 expression	Category	N0	N1	N2	p-value
IRS score	Negative (0-1)	5 (62.5%)	8 (61.53%)	5 (18.52%)	p=0.0006338
	Weak (2-3)	3 (37.5%)	4 (30.77%)	8 (29.63%)	
	Moderate (4-8)	0 (0%)	1 (7.69%)	12 (44.44%)	
	Strong (9-12)	0 (0%)	0 (0%)	2 (7.40%)	

Table 4. Spearman correlation test for N cadherin and MMP-9.

	N-cadherin expression	
MMP-9 expression	r _s	0.27
	P	0.0638
	N	48

a lack of noteworthy correlation between N-cadherin and MMP-9 in tumor cells, with a p-value of 0.0638 (p < 0.05) and a correlation coefficient of 0.27 (Table 4).

DISCUSSION

Following lung cancer, laryngeal squamous cell carcinoma takes the position of the second most common malignancy in the respiratory

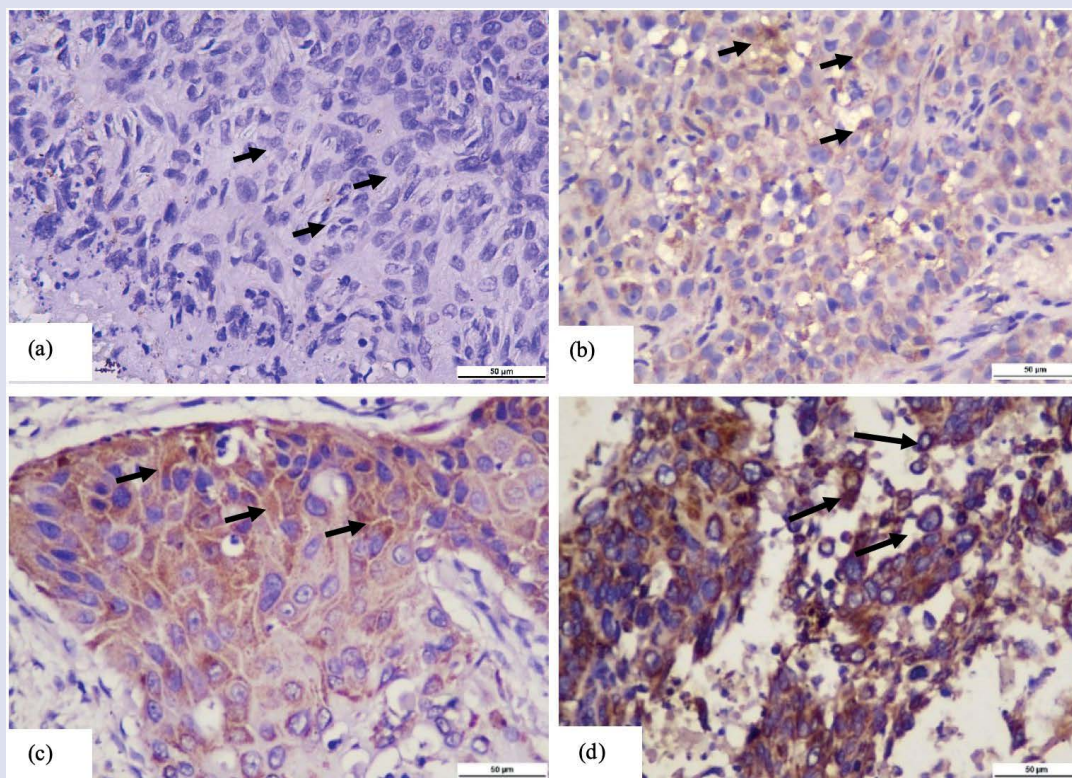


Figure 1. N-cadherin expression at various intensities (black arrow), 400x magnification. (a) N-cadherin is not expressed in N0 (IRS score 0), (b) weak intensity at stage N1 (IRS score 3), (c) moderate intensity at stage N2 (IRS score 6), (d) strong intensity at stage N2 (IRS score 9).

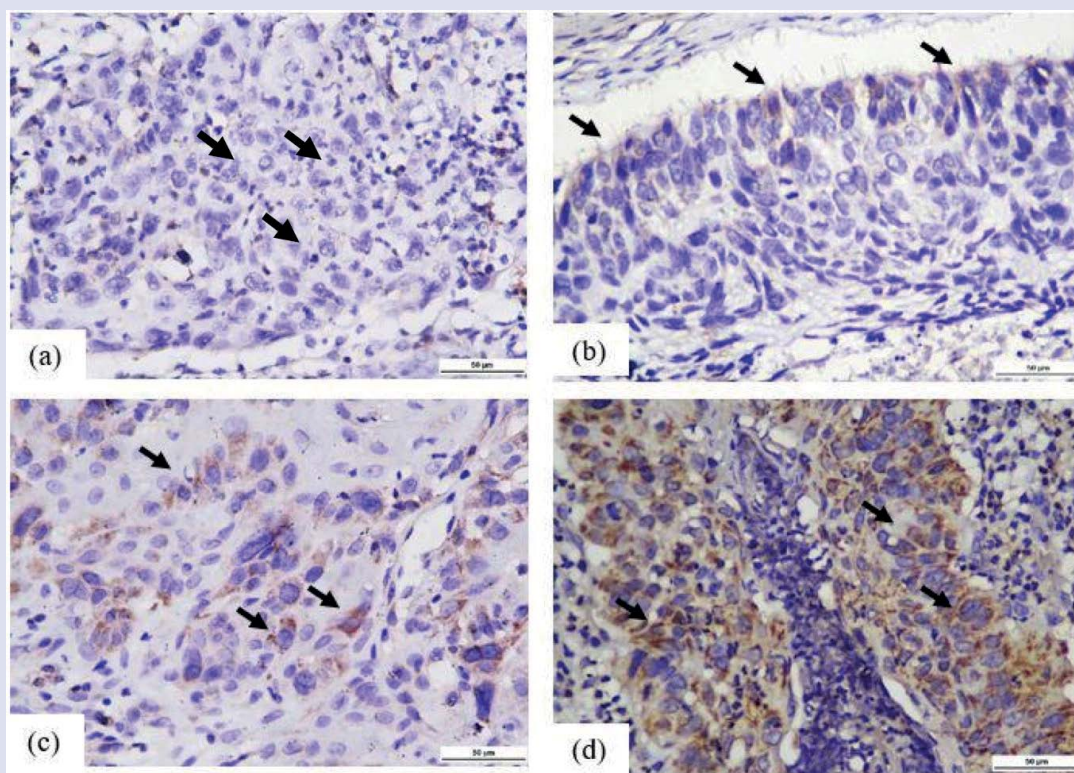


Figure 2. MMP-9 expression at various intensities (black arrow), 400x magnification. (a) MMP-9 is not expressed in N0 (IRS score 0); (b) MMP-9 is expressed with weak intensity in N1 (IRS score 3); (c) MMP-9 is expressed with moderate intensity in N2 (IRS score 6); (d) Tumor expressed with strong intensity in N2 (IRS score 9).

tract.¹ The prognosis of laryngeal carcinoma patients is significantly influenced by nodal metastases. Unfortunately, not all laryngeal carcinoma patients receive neck dissection.⁶ The existence of a preoperative biomarker to predict metastatic status will be very helpful to establish the aggressiveness of therapy and patient prognosis.

Among laryngeal squamous cell carcinoma patients, males constituted the majority (94%), with a male-to-female ratio of 15.7:1, as observed in this study. The ratio observed in this study mirrors that reported in other research^{15,16} and Globocan data.¹⁷ It can be caused by various factors. First, lifestyle differences, where men are more often exposed to alcohol and cigarette smoke, which are the main risk factors for laryngeal carcinoma. Second, there are hormonal and chromosomal factors. The X chromosome contains many immune-related genes and several X-linked microRNAs that can facilitate the modulation of certain immune responses by targeting immune-related genes. This mechanism also underlies many autoimmune diseases that often occur in women.¹⁸ Estrogen levels in women are known to have a protective effect on tumor development.¹⁹

Most patients diagnosed with laryngeal squamous cell carcinoma fell within the age bracket of 51-60 years (37.5%), with an average age of 59.5 years and a range from 27 to 84 years, as indicated by the data from this study. These results are in accordance with WHO and the American Cancer Society.^{1,16,17} Laryngeal carcinoma that occurs in young patients and non-smokers is frequently associated with HPV virus (8-54%).¹⁷

Through immunohistochemical examination, this study evaluated N-cadherin expression in laryngeal squamous cell carcinoma tumors using specific antibodies. Statistical analysis showed that no disparity was observed in N-cadherin expression among tumor cells at different radiologically staged N stages, with p-value = 0.099 (p < 0.05). This is different from a study that meaningful variations and associations were observed in the N-cadherin expression across different N stages assessed in nasopharyngeal carcinoma.¹⁰ The relationship between tumor cell aggressiveness and N-cadherin expression is more pronounced in epithelial carcinoma.⁵ The lack of correlation between N stage and N-cadherin expression indicates the possibility that N-cadherin expression in squamous cell carcinoma of the larynx is more related to cell invasion (T stage), rather than metastasis (N stage). N-cadherin's inability to permeate blood vessel walls may contribute to this circumstance. Endothelial cells create a barrier along the blood vessel walls. The cell bonds found in the endothelium are adherent and tight junctions. Tight junctions are composed of occludin and claudin proteins, while adherent junctions are composed of cadherin, especially Vascular Endothelial cadherin (VE-cadherin) but, not N-cadherin. The loss of bonds between cells will damage to the integrity of the endothelium and allow the tumor to penetrate the vascular wall, which is important to metastatic processes.²⁰

MMP-9 expression was also assessed by immunohistochemical examination with monoclonal antibodies against MMP-9. A significant disparity was observed in MMP-9 expression across different N stages, with a p-value of 0.0006338 (p < 0.05). This is similar to other studies.²¹⁻²³ MMP-9, being a protease enzyme, is involved in numerous physiological and pathological processes, leading to this occurrence. MMP-9 can degrade various proteins, such as gelatin, collagen, and elastin; so it can regulate the process of extracellular matrix remodeling, which involves modifying cellular interactions and interactions between cells and the extracellular matrix.²⁴ An important process in the proliferation and growth of tumor cells is the ability of MMP-9 to degrade type IV collagen. MMP-9 also regulates cancer cell growth, metastasis, angiogenesis, and the immune response to cancer cells. The initiation of tumor cell migration from the primary tumor coincides with the loss of basement membrane continuity.²⁵⁻²⁷ Overexpression of MMP-9 in tumor cells will cause a loss of balance between MMP-9

and TIMP (*Tissue Inhibitor of MMPs*)²⁸ and its impact on lymph node metastases is substantial in patients with laryngeal carcinoma. All of the processes in the metastatic cascade require the destruction of the extracellular matrix structure.^{29,30}

Additionally, MMP-9 contributes to the enhancement of tumor cell migration. Due to the activation of Rho-GTPase, the actin cytoskeleton will undergo polymerization and cause changes in the shape of migrating tumor cells. The locomotion of tumor cells depends on the interaction between integrins and MMP-9. MMP-9 will bind to integrin through its hemopexin domain, attach to the cell surface, and increase tumor cell migration. In addition, this interaction will also induce the synthesis of beta-catenin, which will increase MMP-9 expression.²¹ MMP-9 will help tumor cells survive in circulation by protecting them from immune cells and help the adaptation of tumor cells at their metastatic sites. The increased MMP-9 causes remodeling of the extracellular matrix structure, so the tumor can infiltrate and grow easily in new locations.^{29,31}

Results from this study revealed no notable correlation between N-cadherin and MMP-9 in tumor cells with a p = 0.0638 (p < 0.05) and a positive correlation coefficient of 0.27, which means a pattern emerges wherein elevated N cadherin expression correlates with heightened MMP-9 expression, although not significant. This situation can occur because tumor metastasis can be influenced by many pathways. This proves that there are other pathways, for instance, pathways involving inflammation and hypoxia which produce MMP-9 without activating the N-cadherin pathway.³²

Unfortunately, there are some limitations in this study. First, the staging was determined based on preoperative radiological images and not pathological. This is due to the limited availability of laryngectomy specimens at all stages. Second, this study only combined the relationship between the two proteins and metastasis status, without considering invasion status.

CONCLUSION

MMP-9 serves as a prognostic marker for metastasis to regional lymph nodes, which can negatively affect patient prognosis.

ETHICAL CONSIDERATION

Approval for this study was granted by the Ethical Committee of Dr. Soetomo Regional Public Hospital under ethical clearance number 1196/LOE/301.4.2/2023.

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CONFLICTS OF INTEREST

The authors declare there are no conflicts of interest

AUTHOR CONTRIBUTOR'S

All authors contributed to data analysis, drafting, and revising of the paper and agreed to be responsible for all aspects of this work.

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