R E S E A R C H   A R T I C L E

A Pilot Study on Immunohistochemical Expressions of NF-κB, Cyclin-D1, VEGF, and Cox-2 in Advanced Stage Laryngeal Carcinoma

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Abstract

BACKGROUND: Progression of laryngeal carcinoma can be classified with the clinical staging, however there are different patterns of progressions observed in the patient with the same clinical stage which also affects their prognoses. Therefore biomarkers should be used. Nuclear factor (NF)-κB, Cyclin-D1, vascular endothelial growth factor (VEGF), cyclooxygenase (Cox)-2 have been reported for laryngeal carcinoma. However, it is still unclear how these markers are expressed and correlated in advanced stage laryngeal carcinoma. Therefore current study was conducted to investigate the expressions of NF-κB, Cyclin-D1, VEGF and Cox-2 and their correlations in advanced stage laryngeal carcinoma.

METHODS: Subjects were recruited and laryngeal biopsies were collected, fixed in formalin and prepared for immunohistochemistry. The immunohistochemistry was performed using mouse monoclonal anti-NF-κB p65, anti-Cyclin-D12 anti-VEGF, and anti-Cox-2 antibodies. The immunohistochemistry results were documented and measured using ImmunoRatio. Pearson or Spearman correlation test was used based on the results of Shapiro-Wilk test of normality. A *p*-value of less than 0.05 is considered statistically significant.

RESULTS: Twelve male subjects were included in this study. Expressions of NF-κB, Cyclin-D1, VEGF dan Cox-2 were clearly observed. Mean of NF-κB, Cyclin-D1, VEGF dan Cox-2 IHC expression levels measured with ImmunoRatio were 57.50±20.06%, 45.00±24.31%, 43.33±17.23% and 40.42±16.98%, respectively. There was significant correlation between the expressions of VEGF dan Cox-2 (p=0.031, r=0.622).

CONCLUSION: Since correlation between the VEGF and Cox-2 expressions was statistically significant, VEGF and Cox-2 might have important roles in the growth, invasion and metastasis of laryngeal carcinoma.

KEYWORDS: advanced stage laryngeal carcinoma, immunohistochemistry, NF-κB, Cyclin-D1, VEGF, Cox-2

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Introduction

The laryngeal carcinoma is the second most common head and neck carcinoma after the nasopharyngeal carcinoma. Smoking and alcohol consumption are known as the risk factors for laryngeal carcinoma.(1) In Indonesia, the incidence of laryngeal carcinoma for men and women are 2.0 per 100,000 and 0.2 per 100,000, respectively.(2) Although progression of laryngeal carcinoma can be classified with the clinical staging, however there are different patterns of progressions observed in the patient with the same clinical

stage which also affects their prognoses.(3) Therefore biomarkers should be used. Various factors, including hypoxia, cytokines, oncogenes, tumor suppressor genes, have been known to regulate the expression of angiogenic factor in laryngeal carcinoma. Nuclear factor (NF)-κB is a transcription factor which control gene expression and regulate the immune response. The activation of the NF-κB affects cancer markers and inflammatory diseases through the process of gene transcription in cell proliferation, survival, angiogenesis, inflammation, tumor promotion and metastasis.(4-6)

Cyclin-D1 plays a role in the cell cycle and cell proliferation and act as an oncogene. There are several studies that have shown a significant association between the increase in the Cyclin-D1 expression and the local tumor extension. NF-κB-mediated continuous cell proliferation can activate Cyclin-D1 via cell cycle modulation.(7,8) Cyclin-dependent kinase (CdK)4 and/or 6 are activated by Cyclin D in the early gap (G)1 phase of the cell cycle.

The other factor is the vascular endothelial growth factor (VEGF), an angiogenic factor, which has specific mitogenic activity for endothelial cells. It is the most important angiogenic factor in the angiogenesis process, both in physiological and pathological conditions.(9) The studies have shown that the overexpression of VEGF was associated with tumor progression.(9-11)

Cyclooxygenase (Cox)-2 has been suggested as a better marker for tumor promotion and metastasis. Cox-2 was identified as a metastatic factor in bone, lymph nodes, liver, brain and other organs.(12) The Cox-2 expression can be used as a predictive and prognostic marker of laryngeal squamous cell carcinoma.(13,14)

Although NF-κB, Cyclin-D1, VEGF and Cox-2 have been reported as important markers in laryngeal carcinoma, it is still unclear how these markers are expressed and correlated in advanced stage laryngeal carcinoma. Not enough information can be gathered, meanwhile correlations of these markers should be important to give added value for the improvement of clinical evaluation. Therefore current study was conducted to investigate the expressions of NF-κB , Cyclin-D1, VEGF and Cox-2 and their correlations in advanced stage laryngeal carcinoma.

Methods

Subject Recruitment and Selection
Subjects were recruited from January 1, 2018 to June 30, 2020, in the Ear, Nose & Throat (ENT) Clinic of Dr. Saiful

Anwar Hospital, Malang. A written Informed consent was obtained from all subjects.

The subjects' demography such as gender, age, smoking history and clinical stage were recorded. The clinical staging was determined based on the American Joint Committee of Cancer (AJCC).(15) Laryngeal biopsies of subjects with advanced stage laryngeal squamous cell carcinoma were included. However biopsies of the subjects who underwent radiotherapy or chemotherapy were excluded. This study was approved by The Ethics Committee of Faculty of Medicine, Universitas Brawijaya (No. 100/092/K.3/302/2020).

Immunohistochemistry (IHC)
Formalin-fixed and paraffin-embedded tissues were processed in the Laboratory of Anatomic Pathology, Dr. Saiful Anwar Hospital, Malang. The tissues were sliced with microtome, deparaffinized, antigen-retrieved, endogenous-peroxidase-blocked and applied with blocking solution. Mouse monoclonal anti-NF-κB p65 (sc-514451, Santa Cruz, Dallas, TX, USA), anti-Cyclin-D1 (sc-8396, Santa Cruz), anti-VEGF (sc-7269, Santa Cruz), and anti-Cox-2 (sc-376861, Santa Cruz) antibodies were applied as primary antibodies. Bounds of antigen-antibody were further processed with N-Histofine Simple Stain MAX PO containing polymer anti-mouse and anti-rabbit secondary antibodies, peroxidase and 3,3′-diaminobenzidine buffer (Nichirei, Tokyo, japan). The immunohistochemistry results were documented, five fields were selected for each sample, under an upright light microscope (Nikon, Tokyo, Japan). Documented results were measured using ImmunoRatio.

Statistical Analysis
Correlation analysis of NF-κB and Cyclin-D1, VEGF and Cox-2 expression were conducted using SPSS for Windows software version 20 (IBM, Armonk, NY, USA). Pearson or Spearman correlation test was used based on the results of Shapiro-Wilk test of normality. A p-value of less than 0.05 is considered statistically significant.

Results

Twelve male subjects were included in this study. Most of the subjects were older adult (56-65 years) (50%), had history of smoking (91.67%), tumor size of T4 (66.67%), nodule staging of N2 (66.7%) and stadium of IVa (75%) (Table 1).

Table 1. Advanced laryngeal carcinoma subject characteristics (n=12).

Characteristics	n	%
Gender		
Male	12	100
Female	0	0
Age Group		
Young Adult (36-45 years)	2	16.67
Middle Adult (46-55 years)	1	8.33
Older Adult (56-65 years)	6	50
Elderly (> 65 years)	3	25
Smoking History	11	91.67
Clinical Staging		
Tumor		
T1	0	0
T2	2	16.67
T3	2	16.67
T4	8	66.67
Nodule		
N0	3	25
N1	0	0
N2	8	66.67
N3	1	8.33
Stadium		
III	2	16.67
IVa	9	75
IVb	1	8.33

IHC Expressions of NF-κB, Cyclin-D1, VEGF and Cox-2

Expressions of NF-κB, Cyclin-D1, VEGF dan Cox-2 were clearly observed. Brown color in the cytoplasm was documented for NF-κB (Figure 1A). Brownish smear color in the cytoplasm was documented for VEGF dan Cox-2 (Figure 1C&1D). Meanwhile brown color in the nucleus was documented for Cyclin-D1 (Figure 1B). Mean of NF-κB, Cyclin-D1, VEGF dan Cox-2 IHC expression levels measured with ImmunoRatio were 57.50±20.06%, 45.00±24.31%, 43.33±17.23% and 40.42±16.98%, respectively (Figure 1E).

Correlation of NF-κB, Cyclin-D1, VEGF and Cox-2 IHC Expressions

After being analysed with Spearman correlation test, almost all correlations between the NF-κB, Cyclin-D1, VEGF and Cox-2 were not significant, except the correlation between VEGF dan Cox-2 ($p=0.031$, $r=0.622$) (Table 2).

Discussion

Current results showed that advanced stage laryngeal carcinoma had >50% NF-κB expression level. This result is in accordance with previous study showing that normal tissue has <5% NF-κB expression level, dysplastic tissue has 5-50% NF-κB expression level, and carcinoma tissue has >50% NF-κB expression level.[16]

For Cyclin-D1, low expression was categorized as ≤25% and high expression was >25%.[17] In current results, Cyclin-D1 expression level of advanced stage laryngeal carcinoma was 45.00±24.31%, suggesting a confirmation of high Cyclin-D1 expression.

For VEGF, the expressions were classified into level 0 (<1% positive cells), level 1 (>1-33% positive cells), level 2 (>34-66% positive cells) and level 3 (>67% positive cells).[17] In current results, VEGF expression level was 43.33±17.23%, suggesting the advanced stage laryngeal carcinoma also had high expression of VEGF.

For Cox-2, the negative and positive cut-off was reported as 10%, therefore ≤10% was negative while >10% was positive.[18] In current result, the Cox-2 expression level was 40.42±16.98%, suggesting high expression of Cox-2 as well in the advanced stage laryngeal carcinoma.

Modulation of VEGF by prostaglandins produced by Cox-2 or induction of endothelial cells by Cox-2 can lead to tumor angiogenesis.[19] In the current results, VEGF and Cox-2 expressions of the advanced stage laryngeal carcinoma were significantly correlated ($r=0.622$, $p=0.031$). These results are similar with a previous report showing that a positive correlation between the VEGF and Cox-2 expressions in laryngeal squamous cell carcinoma ($r=0.756$, $p<0.01$).[20] There was a higher increase in the expression of VEGF and Cox-2 in advanced laryngeal squamous cell carcinoma (stadium 3 and 4) than early stadium (stadium 1 and 2).[21] This suggested the major role of VEGF and Cox-2 in the growth, invasion and metastasis of laryngeal carcinoma.

The limitation of available sample numbers in this study which were collected in the last 2 years should be improved, therefore advanced stage laryngeal carcinoma samples would be collected continuously for future investigation.
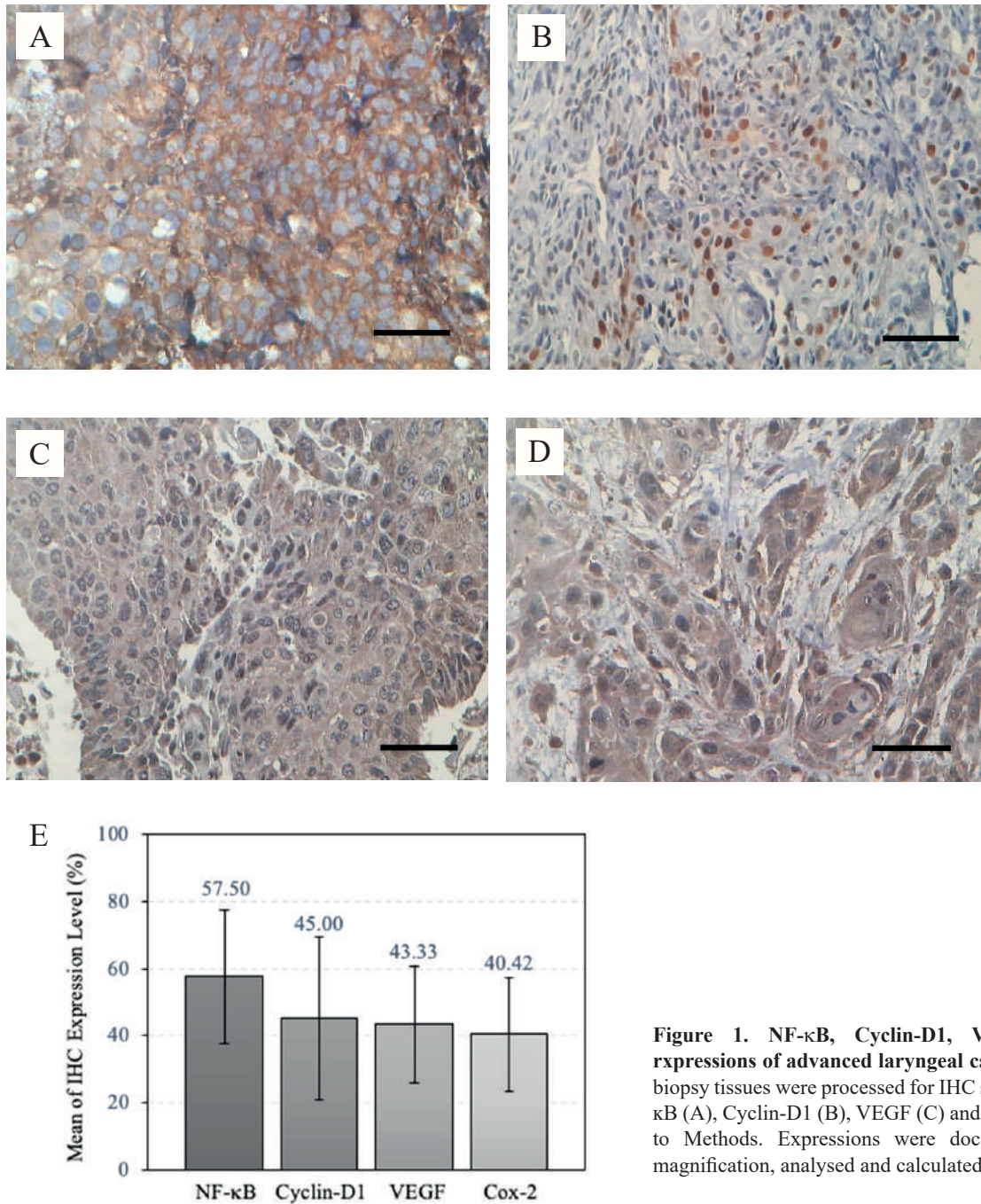
Figure 1. NF-κB, Cyclin-D1, VEGF, and Cox-2 rxpressions of advanced laryngeal carcinoma. Laryngeal biopsy tissues were processed for IHC staining to detect NF-κB (A), Cyclin-D1 (B), VEGF (C) and Cox-2 (D) according to Methods. Expressions were documented with 400x magnification, analysed and calculated. Black bar: 10 μm.

Table 2. The correlation between NF-κB , Cyclin-D1, VEGF and Cox-2 IHC expressions of advanced laryngeal carcinoma.

Biomarker	NFκB		Cyclin D1		VEGF		Cox-2	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
NFκB	-	-	-0.101	0.755	0.182	0.572	-0.142	0.661
Cyclin-D1	-0.101	0.755	-	-	0.106	0.743	0.156	0.628
VEGF	0.182	0.572	0.106	0.743	-	-	0.622	0.031*
Cox-2	-0.142	0.661	0.156	0.628	0.622	0.031*	-	-

Conclusion

Since correlation between the VEGF and Cox-2 expressions was statistically significant, VEGF and Cox-2 might have important roles in the growth, invasion and metastasis of laryngeal carcinoma. Further investigation with more numbers of sample should be pursued.

Acknowledgements

Authors Contribution

PR contributed in research conception and design, ATW, FS, SK, and KN performed the data acquisition and analysis, result interpretation, manuscript preparation, figure and table design. FS, HTN, EH, ADW, HS and HSY contributed in data analysis and result interpretation. FS and NS contributed in data analysis. All authors took parts in giving critical revision of the manuscript.

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