RESEARCH ARTICLE

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Exploring the Relationship Between *YAP* (Yes-Associated Protein) and *VEGF-A* with Distant Metastasis in Nasopharyngeal Carcinoma

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Abstract

Objective: To investigate the relationship between Yes-Associated Protein (*YAP*) and Vascular Endothelial Growth Factor-A (*VEGF-A*) in nasopharyngeal carcinoma (NPC) and evaluate their potential as predictive biomarkers for distant metastasis. **Methods:** This observational analytic study was conducted from May to August 2023, including 60 NPC cases diagnosed histopathologically at the Department of Anatomical Pathology, Faculty of Medicine, Universitas Indonesia/Cipto Mangunkusumo Hospital. The study involved 30 NPC cases with distant metastasis and 30 without distant metastasis. Immunohistochemical analysis was performed using primary antibodies (rabbit *YAP*, D8H1X; mouse VEGF, clone C-1 sc-7269). Expression levels of *YAP* and *VEGF-A* were semi-quantitatively assessed using the H-score formula on 500 tumor cells. Cutoff points for *YAP* and *VEGF-A* were determined through Receiver Operating Characteristic (ROC) curve analysis and the Youden index. Statistical analyses, including Chi-square tests, were conducted using SPSS 25.0 to evaluate the relationship between marker expression and the presence of distant metastasis. **Results:** A significant difference in *YAP* expression was observed between NPC cases with distant metastasis (80%) and those without (23.3%, $p<0.001$). Similarly, *VEGF-A* expression was notably higher in cases with distant metastasis (86.7%) compared to those without (20%, $p<0.001$). A significant association between *YAP* and *VEGF-A* expression was found ($p=0.001$). ROC curve analysis showed that *YAP* (AUC=0.738, 95% CI 0.602-0.874) and *VEGF-A* (AUC=0.842, 95% CI 0.735-0.950) effectively predict distant metastasis. The double co-high-expression group (elevated *YAP* and *VEGF-A*) exhibited a significantly higher rate of distant metastasis compared to the non-double co-high-expression group ($p<0.001$). **Conclusion:** Elevated expressions of *YAP* and *VEGF-A* are significantly associated with distant metastasis in NPC, suggesting their potential as biomarkers for predicting metastatic risk.

Keywords: Distant metastasis- Hippo- nasopharyngeal carcinoma- *VEGF-A*, *YAP*

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Introduction

Nasopharyngeal carcinoma (NPC), a malignant epithelial neoplasm characterized by squamous differentiation, is prevalent in Southeast China and remains an endemic disease in several Asian and North African countries [1–3]. NPC instances in Indonesia are ranked as the second-largest in the country, following China [4]. The disease exhibits a higher prevalence among males, specifically between the ages of 40 and 60 [1, 5].

Approximately 80% of (NPC) patients are detected at an advanced local stage, with 10% presenting with distant metastasis at the time of diagnosis [4]. This is attributed to NPC's asymptomatic nature in its early stages and high propensity for metastasis, facilitated by the abundant lymphatic flow in the nasopharynx [4, 6, 7]. The 5-year survival rate drops from 82% in the early stages to 49% with distant metastasis [8]. Given the increased mortality risk associated with metastasis, identifying biomarkers for prognostic prediction is crucial. Research on NPC biomarkers is imperative, but understanding molecular mechanisms and signaling pathways related to NPC metastasis remains challenging [4].

Vascular Endothelial Growth Factor (*VEGF*), known for stimulating angiogenesis, is associated with NPC patient survival [9, 10]. Some studies also identify another role of *VEGF* as a marker for metastasis in NPC [10, 11]. *VEGF-A*, a member of the *VEGF* family, is implicated

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in angiogenesis and lymphangiogenesis, yet its direct relationship with distant NPC metastasis is underexplored [9, 12].

In addition to *VEGF*, downstream components of the *VEGF*-activated Hippo signaling cascade have garnered considerable attention [13, 14]. Dysregulation of this pathway prompts the accumulation of Yes-Associated Protein (*YAP*) in the nucleus, initiating the transcription process that stimulates tumor cell proliferation, migration, and epithelial-mesenchymal transition (EMT) [14–18]. Notwithstanding the enigmatic nature of *YAP*'s function in NPC and its correlation with distant metastasis, the objective of this study is to elucidate the relationship between *YAP* and *VEGF-A* expression, as well as the cumulative impact of the two on the prevalence of distant metastasis in NPC. This study also represents a significant advancement in validating *YAP* expression as a robust prognostic indicator for distant metastasis in NPC.

Materials and Methods

This study utilized an observational analytic cross-sectional design from May to August 2023. Ethical approval, a waiver of informed consent, and location licensing were obtained before initiation. The research centered on cases of locally advanced and advanced-stage NPC, diagnosed histopathologically at the Department of Anatomical Pathology, Faculty of Medicine, Universitas Indonesia/Cipto Mangunkusumo Hospital, during the period from January 2018 to December 2021. The sample size consisted of 60 cases, involving 30 with distant metastasis and 30 without, chosen through consecutive sampling. Distant metastasis status was determined through radiological examinations (chest/bone x-ray, abdominal ultrasound, bone scan, chest/bone/abdominal CT scan, and MRI). Patients diagnosed with stages III and IVA NPC, according to the AJCC 8th edition TNM classification, were selected for the case group without distant metastasis. Exclusion criteria included double primary and residual patients. Clinical and histopathological data, including gender, age, smoking habits, signs and symptoms, stage, metastasis location, histopathology type, and lymphovascular invasion, were recorded.

Immunohistochemical analysis utilized primary antibodies (rabbit *YAP*, D8H1X, diluted at 1:200; mouse *VEGF* clone C-1 sc-7269, diluted at 1:100). *YAP* and *VEGF-A* expression, semi-quantitatively assessed through the H-score formula on 500 tumor cells, categorized results based on a cutoff point determined from the Receiver Operating Characteristic (ROC) curve and Youden index [19]. Positive *YAP* expression was determined by a brown appearance in the nucleus or both the nucleus and cytoplasm. Positive *VEGF-A* expression was identified by a brown appearance in the cytoplasm. An Area Under the Curve (AUC) value >0.6 was considered indicative of a reliable model [20]. Statistical analyses involved Chi-square tests for expressions of *YAP*, *VEGF-A*, and their co-expression with distant metastasis status in NPC. A p-value <0.05 was considered significant.

Results

Patient characteristics

The clinical characteristics of the 60 cases are summarized in Table 1. Samples were allocated into two groups, particularly focusing on the metastasis status. As can be seen from Table 1, predominantly, the male gender accounts for 68.3% of the cases, while those aged 47 years or younger and non-smokers account for 56.7% and 58.3%, respectively. The results obtained from this study also depict the most common clinical presentations involving neck lumps (81.7%), hearing impairment (71.7%), and pain (70%). NPC is often diagnosed at advanced stages, with T4 (53.3%) and N3 (50%) being prevalent and distant metastasis most frequently observed in the bones (83.3%). The histopathological analysis reveals that non-keratinizing squamous cell carcinoma (NK-NPC) is the most prevalent, accounting for 96.7% of cases. Moreover, it is worth noting that there were no lymphovascular invasions in all cases in this study.

Expression of YAP and VEGF-A in NPC with and without distant metastasis

Immunohistochemistry examination revealed that *YAP* consistently exhibited positive expression in the nucleus and cytoplasm of neoplastic cells without exclusive nuclear positivity (Figure 1). *VEGF-A* was expressed in the cytoplasm of cancer cells, as shown in Figure 2. The semi-quantitative H-score analysis method was used to classify expressions into negative, weak positive, moderate positive, and strong positive categories.

To establish the basis for the remarkable relationship between *YAP* and *VEGF-A* and the metastasis status of NPC, we employed the ROC curve method to identify the most significant *YAP* and *VEGF-A* expression cut-off point as a predictor marker of distant metastasis in NPC (Figure 3). The ROC curve analysis reveals that both *YAP* and *VEGF-A* exhibit considerable precision in predicting distant metastasis of NPC. It is apparent from the AUC that values for *YAP* and *VEGF-A* were 0.738 (95% CI 0.602-0.874) and 0.842 (95% CI 0.735-0.950), respectively. In further analysis, we determined the optimum sensitivity and specificity metrics for each marker to establish optimal cut-off values for *YAP* and *VEGF-A*. *YAP* was set at 89.8, achieving a sensitivity of 80% and a specificity of 76.7%. For *VEGF-A*, the optimal cut-off was determined to be 183.2, demonstrating a sensitivity of 86.7% and a specificity of 80%.

We subsequently separated the cases into the high and low expression of *YAP* and *VEGF-A* based on the ROC analysis cut-off point. As shown in Table 2, statistical analysis revealed that a higher proportion of patients with distant metastasis (80%) had a significantly higher *YAP* expression than those without distant metastasis (23.3%). Similarly, a higher proportion of patients with distant metastasis (86.7%) had high *VEGF-A* expression compared to those without distant metastasis (20%) with statistical significance. Increasing *YAP* expression was 13.1 times more frequent (95% CI 3.8-45) in the NPC group with distant metastasis, while elevating *VEGF-A* expression was 26 times more frequent (95% CI 6.5-

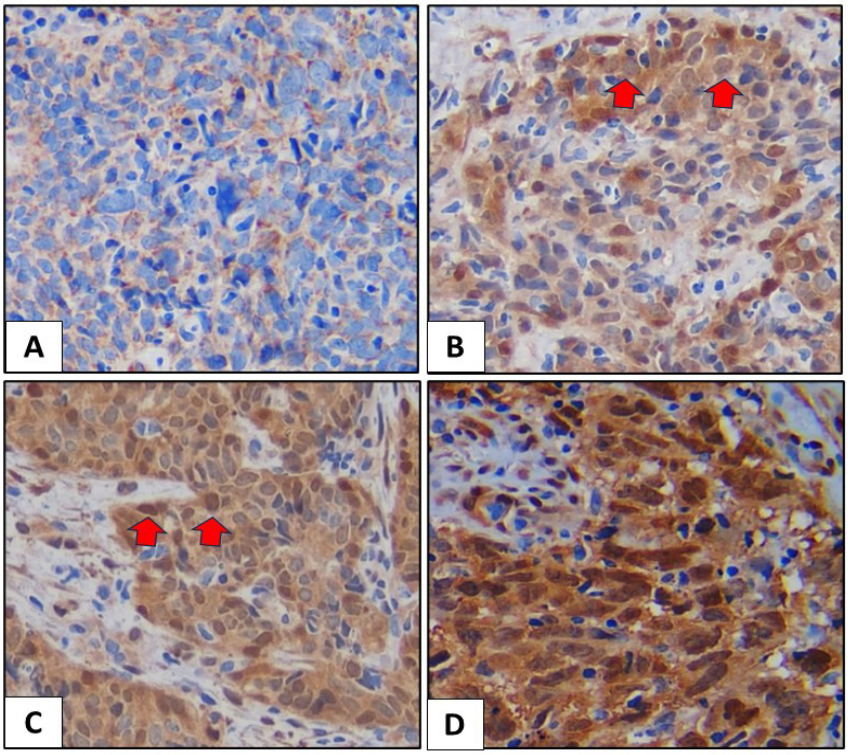

Figure 1. *YAP* Immunohistochemistry. A, Predominantly negative expression of *YAP* (400x magnification); B, Weak positive expression of *YAP*, indicated by the arrow (400x magnification); C, Moderately positive expression of *YAP*, indicated by the arrow (400x magnification); D, Predominantly strong positive expression of *YAP* (400x magnification).

103.4).

We endeavored to elucidate the relationship between the expression of *YAP* and *VEGF-A* by examining the distant metastasis status of NPC variables. Subsequently, we sought to enhance the comprehensiveness of our study by obtaining detailed data on the statistical correlation between *YAP* and *VEGF-A* (Table 3). The table presented herein illustrates the NPC patients who exhibited increasing *YAP* levels and concomitantly displayed elevated expression of *VEGF-A* ($p = 0.001$).

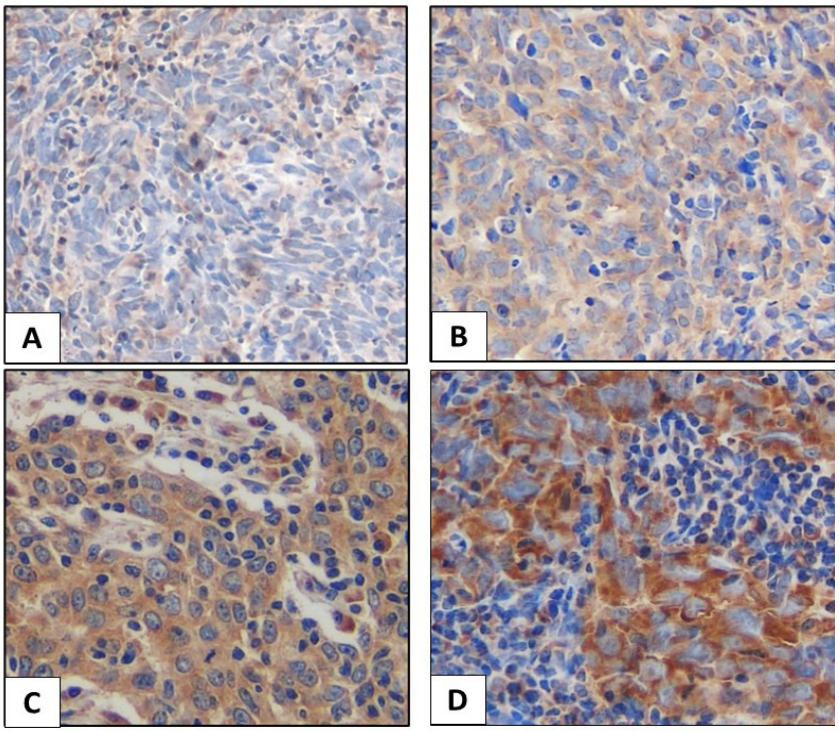

Figure 2. *VEGF-A* Immunohistochemistry. A, Predominantly negative expression of *VEGF-A* (400x magnification); B, Weakly positive dominant expression of *VEGF-A* (400x magnification); C, Moderately positive dominant expression of *VEGF-A* (400x magnification); D, Predominantly strong positive expression of *VEGF-A* (400x magnification).

Table 1. Demographic and Histomorphological Characteristics of the Study Sample

Characteristics	Amount (%)	Distant metastasis	
		Yes n (%)	No n (%)
Age			
≤ 47 years	34 (56.7)	19 (55.9)	15 (44.1)
> 47 years	26 (43.3)	11 (42.3)	15 (57.7)
Gender			
Male	41 (68.3)	21 (51.2)	20 (48.8)
Female	19 (31.7)	9 (47.4)	10 (52.6)
Smoking habits			
Yes	24 (40)	13 (54.2)	11 (45.8)
No	35 (58.3)	17 (48.6)	18 (51.4)
No data	1 (1.7)	0	1 (100)
Sign and symptoms			
Neck lump	49 (81.7)	29 (59.2)	20 (40.8)
Visual impairment	21 (35)	10 (47.6)	11 (52.4)
Hearing impairment	43 (71.7)	21 (48.8)	22 (51.2)
Olfactory impairment	23 (38.3)	12 (52.2)	11 (47.8)
Neurological disorder	25 (41.7)	12 (48)	13 (52)
Pain	42 (70)	20 (47.6)	22 (52.4)
Bone/joint pain	11 (18.3)	6 (54.5)	5 (45.5)
Stage T			
T1	1 (1.7)	0	1 (100)
T2	17 (28.3)	10 (58.8)	7 (41.2)
T3	10 (16.7)	4 (40)	6 (60)
T4	32 (53.3)	16 (50)	16 (50)
Stage N			
N0	5 (8.3)	0	5 (100)
N1	6 (10)	1 (16.7)	5 (83.3)
N2	19 (31.7)	7 (36.8)	12 (63.2)
N3	30 (50)	22 (73.3)	8 (26.7)
Stage			
III	10 (16.7)	0	10 (100)
IVA	20 (33.3)	0	20 (100)
IVB	30 (50)	30 (100)	0
Distant metastasis location			
Bone	25 (83.3)		
Liver	6 (20)		
Lung	6 (20)		
Brain	2 (6.6)		
Histopathological type			
Keratinizing squamous cell carcinoma	2 (3.3)	0	2 (100)
Non-keratinizing squamous cell carcinoma	58 (96.7)	30 (51.7)	28 (48.3)
Lymphovascular invasion			
Yes	0	0	0
No	60 (100)	30 (50)	30 (50)

Co-expression of YAP and VEGF-A in NPC with and without distant metastasis

Furthermore, we then analyze the potential of combining two markers in predicting distant metastasis in NPC. Our finding reveals that distant metastasis cases were significantly higher in double co-high expression (elevated *YAP* and *VEGF-A*) than others (non-double co-high expression), p<0.001.

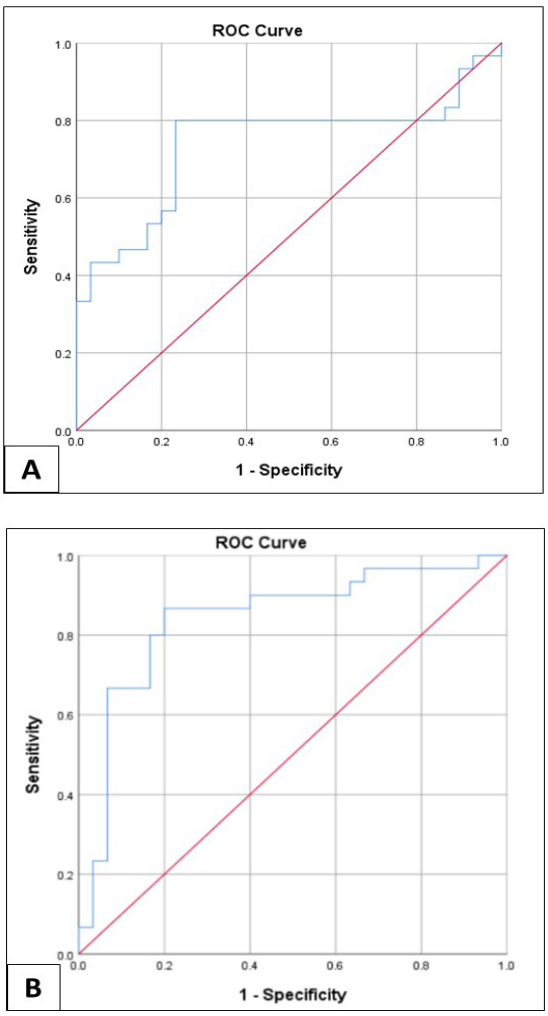

Figure 3. ROC Curve of *YAP* (A) and *VEGF-A* (B) Expression to Predict the Incidence of Distant NPC Metastasis.

Discussion

In this research, the majority of the samples were male, consistent with the findings of Salehiniya et al [5], who reported a 2-3 times higher prevalence of NPC in men compared to women. This difference is attributed to factors such as smoking, a well-known risk factor for NPC [5]. Long et al [21] indicated a 56-59% higher NPC risk in smokers. However, a significant portion of our sample did not smoke, possibly due to population differences and recall bias [21]. The predominant age group was ≤47 years, consistent with Toumi et al [7]. WHO data emphasize NPC occurrence in individuals under 50 in high-incidence areas like Indonesia [1].

Common symptoms included neck lumps, correlating with literature attributing 70% of neck lumps to retropharyngeal lymph node enlargement, the primary drainage station for the nasopharynx, with up to 60% of

Table 2. Expression of *YAP, VEGF-A* in NPC with and without Distant Metastasis

		Distant metastasis		p	OR
		Yes (n=30)	No (n=30)		(CI 95%)
YAP expression	High	24 (80%)	7 (23.3%)	<0.001*	13.1
	Low	6 (20%)	23 (76.7%)		(3.8-45)
VEGF-A expression	High	26 (86.7%)	6 (20%)	<0.001*	26
	Low	4 (13.3%)	24 (80%)		(6.5-103.4)

Table 3. Correlation *YAP* and *VEGF-A* Expression

		YAP expression		p
		High	Low	
VEGF-A expression	High	23	9	0.001*
	Low	8	20	

NPC cases showing spread to these nodes [1, 22]. Most samples were diagnosed at stages T4 and N3, with bone being the most common site for distant metastasis, which aligns with previous studies [4, 7, 23]. NPC often remains asymptomatic in the early stages, leading to late-stage diagnoses, compounded by its high metastatic tendency due to rich lymphatic flow in the nasopharynx [1, 4, 6, 7]. Guo et al. [24] and Chan et al. [1] mentioned that distant NPC metastasis can occur in bones, lungs, liver, and the brain. Brain metastasis in NPC is very rare, consistent with the findings in this research [24]. The pathomechanism is associated with the "seed and soil" theory, indicating that the interaction between NPC tumor cells and the microstructure environment in the brain does not occur optimally [24].

The most common histopathological type in this study is NK-NPC, and all samples lacked lymphovascular invasion. Similar findings were reported in studies conducted in Indonesia, China, and North Africa, where NK-NPC was the predominant type [7, 25, 26]. NK-NPC is prevalent in NPC-endemic areas like Indonesia [1]. Duprez et al. [27] investigating distant metastasis in head and neck cancer, found no direct relationship between lymphovascular invasion and metastasis. The low incidence of lymphovascular invasion in our biopsy-based study may not fully represent invasion in non-biopsied tumor areas.

In this study, positive *YAP* expression was consistently observed in both the nucleus and cytoplasm of tumor cells. The dysregulation of the Hippo signaling pathway is known to cause *YAP* translocation from the cytoplasm to the nucleus, contributing to the development of distant metastasis [18, 28]. To capture both actively working (nuclear) and potentially working (cytoplasmic) *YAP*, we selected primary antibodies capable of detecting both aspects.

Shin et al. [29] noted that the location of *YAP* positivity varies in head and neck squamous cell carcinoma (SCC) cases. For example, well-differentiated oral SCC exhibits positivity in the nuclei, while poorly differentiated oral SCC shows positivity in the nucleus or cytoplasm [30]. Cytoplasmic *YAP* may result from gene amplification, leading to excessive expression in both cellular compartments [30]. Similar findings were observed in NPC patients with hepatitis B co-infection, where *YAP1* positivity was found in both the nuclei and cytoplasm of tumor cells [17]. Sakabe et al. [31] suggested another role of cytoplasmic *YAP* in angiogenesis and vascular remodeling, particularly associated with endothelial cell proliferation and migration. Cervical cancer shows diversity in *YAP* positivity, with SCCs exhibiting cytoplasmic *YAP*, while adenocarcinomas show nuclear and cytoplasmic positivity [32]. A meta-analysis by Sun et al. [33] concluded that both nuclear and nuclear-cytoplasmic *YAP* positivity is associated with poor prognosis in carcinoma patients.

Our research found a significant difference in *YAP* expression between NPC groups with and without distant metastasis. Previous studies evaluating the effect of inhibiting *YAP1* activity resulted in reduced proliferation, migration, and invasion of NPC cells while increasing E-cadherin expression and decreasing N-cadherin and Vimentin expression [17]. Hence, it can be conceivably hypothesized that the oncogenic role of *YAP1* is attributed to regulating tumor cell proliferation and EMT, influencing the metastatic process [17]. In contrast, Yuan et al. [34] reported conflicting results in breast carcinoma, where negative *YAP* expression was associated with the loss of the *YAP* gene locus [34]. They suggested *YAP* acts as a tumor suppressor through its interaction with p53-binding protein-2, involved in cell apoptosis [34].

The dual nature of *YAP*, exhibiting both oncogenic and tumor-suppressive characteristics, may be influenced by epigenetic modifications and variations in *YAP* binding partners in the nucleus [35]. Our study supports the oncogenic role of *YAP*, demonstrated by satisfactory AUC values, sensitivity, and specificity for *YAP*, along with notable differences in expression between NPC groups with and without distant metastasis.

The expression of *VEGF-A* in our study was identified in the cytoplasm of tumor cells, highlighting a significant distinction between the NPC group with and without distant metastasis. Similar findings were reported by Li et al. [36], suggesting a significant association between *VEGF* expression and distant metastasis and recurrence, with no significant correlations with gender, age, T stage, or N stage. The mechanism, as clarified by Chen et al. [11], illustrates that suppressing *VEGF* hinders cell migration, enhances E-cadherin, and reduces N-cadherin, Vimentin, MMP2, and MMP9 [11].

The role of *VEGF* in distant metastasis is also related to its function as an angiogenesis stimulus, both in primary and distant organs [9, 37]. In primary organs, *VEGF* stimulates angiogenesis for tumor growth, while in distant organs, *VEGF* is immunosuppressive and triggers

the transition of tumor cells from dormant to proliferative states [9]. *VEGF* expression is also associated with a higher incidence of lung metastasis in NPC, as supported by studies by Guang Wu et al. [38] and Li et al. [36], which shows a correlation between *VEGF* expression and advanced stages, locoregional recurrence, poor prognosis, and the incidence of distant metastasis in NPC. Furthermore, *VEGF* has been proven to be associated with distant metastasis and poor prognosis in ovarian cancer, breast cancer, and osteosarcoma [11].

In this study, a significant association was identified between increased *YAP* expression and elevated *VEGF-A* levels (p=0.001). These results reflect those of Azad et al [13], who also found that the Hippo signaling pathway is a crucial mediator of angiogenesis. The interaction between *VEGF* ligands and their receptors activates the PI3K and MAPK/ERK signaling pathways, leading to the inactivation of the Hippo pathway and consequent activation of *YAP* [13]. Gong et al. [39], also found a significant positive correlation between *YAP* and *VEGF* expression in hepatoblastoma patients, speculating that heightened *YAP* expression induces increased *VEGF* levels, resulting in enhanced micro-vessel density and angiogenesis, associated with distant metastasis and reduced survival. Our study demonstrates that the incidence of distant metastasis in NPC is 79.7 times higher (OR 79.7, 95% CI 9.2-685.6) in the double co-high-expression group compared to the non-double high-expression group. This finding supports the hypothesis that *YAP* and *VEGF-A* may synergistically contribute to triggering distant metastasis in NPC.

This study is limited by its cross-sectional design, which hinders a detailed understanding of the relationship between *YAP*, *VEGF-A*, and distant metastasis. Though more robust, cohort studies would require an extended data collection period. Furthermore, experimental and in vitro study design is necessary to dissect the putative mechanism of the interaction of *YAP* and *VEGF-A* in regulating NPC metastasis.

Taken together, what emerges from our study reveals that significant differences in *YAP* and *VEGF-A* expressions between NPC groups with and without distant metastasis and higher levels of both markers in the group with distant metastasis suggest their potential as biomarkers for predicting distant metastasis in NPC.

Author Contribution Statement

LW, ML, FH, YDB: Conceptualization, Visualization, Software. LW, ML, FH, RC, TS, YDB: Methodology, Formal Analysis, Resources. LW, ML, YDB: Investigation. LW, ML, RC, TS: Data Curation. LW, ML, FH: Writing – Original Draft, Writing – Review & Editing. LW, ML: Project Administration. LW, RC: Funding Acquisition. LW, RC, TS: Validation. LW, RC, TS, YDB: Supervision.

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Ethical Declaration

This study was approved by the Ethical Committee of Universitas Indonesia/Cipto Mangunkusumo Hospital (Ethical Approval Number: [KET-850/UN2.F1/ETIK/PPM.00.02/2023]).

Informed Consent

This study utilizes data from stored materials, and the need for direct consent has been waived by the Health Research Ethics Committee of the Faculty of Medicine, Universitas Indonesia/Cipto Mangunkusumo Hospital (Waiver Statement Number: [ND-542/UN2.F1/ETIK/PPM.00.02/2023]).

Availability of Data

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Conflict of Interest

The authors declare no conflicts of interest related to this study.

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