

Unveiling the interplay of *YAP1*-driven pathways and miR-340-5P expression: insights into nasopharyngeal cancer metastasis

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Abstract. - **OBJECTIVE:** Nasopharyngeal carcinoma (NPC) is a prevalent malignancy in Southeast Asia and Southern China, with a notable incidence in Indonesia. This study aimed to characterize the expression and correlation of Yes-associated protein (*YAP1*) and miR-340-5p in NPC metastasis tissues.

MATERIALS AND METHODS: This study utilized clinical samples from primary tumors of NPC patients to investigate the expression of *YAP1* and miR-340-5p. The Cancer Genome Atlas (TCGA) Head and Neck Cancer dataset was analyzed to assess *YAP1* and miR-340-5p expression in broader head and neck cancer samples. Protein-protein interaction (PPI) and functional enrichment analyses were performed to understand the putative regulatory mechanisms of *YAP1* and miR-340-5p head and neck cancer. *YAP1* mRNA and miR-340-5p level expression were measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR), and statistical analyses were performed to compare the expression of these markers in NPC samples.

RESULTS: Analysis of clinical samples revealed lower expression levels of *YAP1* and miR-340-5p in metastasis NPC cases compared to non-metastatic cases ($p < 0.0001$). *YAP1* and miR-340-5p revealed a negative correlation in metastasis and non-metastasis samples but were statistically insignificant. Additionally, both genes showed significantly lower expression in stage IVB compared to stage II, III, and IVA NPC tissues ($p < 0.0001$). The TCGA dataset showed consistent decreases in *YAP1* and miR-340-5p expression in head and neck cancer tumors as opposed to normal tissues. Functional enrichment and PPI analysis suggested the involve-

ment of the Hippo signaling pathway and other cancer-related pathways in NPC progression.

CONCLUSIONS: The study highlights the under-expressed *YAP1* and miR-340-5p in metastasis tumor cases, suggesting their potential role as a tumor suppressor in NPC.

Key Words:

YAP1, miR-340-5p, Nasopharyngeal carcinoma, Metastasis.

Introduction

Nasopharyngeal carcinoma (NPC) is an epithelial tumor primarily in the lateral pharyngeal recess. It has complex epidemiological characteristics¹, and distinctive geographical distribution². Global cancer statistics from 2020 show that Southern China and Southeast Asia account for more than 75% of NPC cases³. In Indonesia, NPC is one of the most frequent tumors, ranking fourth behind cervical, breast, and skin cancers. It is also considered the most prevalent malignancy in the head and neck region⁴. The pathogenesis of NPC is strongly associated with Epstein-Barr virus (EBV) infection and other pathogenic infections, such as Human Papilloma Virus (HPV) and bacteria. In addition, poor lifestyle behaviors, such as smoking and alcohol consumption, and environmental factors, including carcinogen exposure, contribute to the increased risk of NPC⁵⁻⁷. Molecularly targeted therapy, immunotherapy, and radiation in conjunction with traditional chemothera-

py are the current treatments for NPC. The overall survival rates of NPC patients have increased as a result of these therapy strategies^{7,8}. However, since NPC patients with recurrence or metastatic carcinomas have poor prognoses, resulting in shorter survival duration for NPC patients, exploring novel oncotargets and developing new treatment strategies for NPC is crucial⁹.

Yes-associated protein (*YAPI*) serves as the terminal mediator within the Hippo signaling pathway and regulates organ size by governing cell proliferation and apoptosis^{10,11}. As a potent transcriptional co-activator, *YAPI* fulfills crucial roles in cell development, stem cell maintenance, normal tissue homeostasis, and regeneration. Aberrant and persistent *YAPI* activation is involved in various aspects of cancer progression and related to resistance in cancer therapy. These roles are predominantly associated with a pro-proliferative and pro-survival transcriptional program prompted by interacting with transcriptional enhanced associate domain (*TEAD*) transcription factors¹². The current data associate elevated nuclear *YAPI* levels with unfavorable prognosis in liver cancer and various human cancer types, including non-small cell lung cancer (NSCLC)¹³, gastric cancer¹⁴, and colorectal cancer (CRC)¹⁵, indicating *YAPI* might have the potential to be an oncoprotein. Accordingly, it has been thought that lowering the YAP level and its activity is a direct approach to inhibiting cancer progression; nevertheless, recent research¹⁶ has shown the complexity of this strategy considering the intricate mechanism.

Earlier studies¹⁷ have reported that YAP interacts with the homolog of p53, the p73 tumor suppressor protein, within the nuclei. When DNA damage occurs, this interaction enhances the expression of p53-regulated Apoptosis-Inducing Protein 1 (*p53AIP1*) and BCL2-associated X (*BAX*) as pro-apoptotic genes to act as a response¹⁷. In breast cancer, the level of YAP was likewise absent or reduced, resulting in the inhibition of anoikis and, furthermore, increased cell invasiveness and stimulated migration, although whether these phenotypes were p73-dependent was not investigated¹⁸. YAP exhibits an additional tumor-suppressing activity by altering its binding partner to RUNX family transcription factor 3 (*RUNX3*) instead of *TEAD*¹⁹. Moreover, a recent study²⁰ reported that the absence of YAP expression is associated with small cell lung cancer (SCLC) cells exhibiting rapid ameboid migration and a high metastatic potential. Therefore, the expression of *YAPI* remains a complex

issue and is context-dependent, relying on the types of tumors. It is necessary to comprehend *YAPI*'s expression in a specific context to unravel its role in tumor progression and metastasis, particularly in NPC, where it has not been elucidated.

With roughly 20-24 nucleotides, microRNAs (miRs, miRNAs) are a class of small, non-coding RNAs with the ability to bind to mRNAs to modify gene expressions at post-transcriptional level and further engage in various cellular activities. MiRs can play a dual role, either as inhibitors or promoters in cancer progression, depending on their different regulations. Several studies²¹ suggest that when miRNA's regulation in targeting oncogenes or tumor suppressor genes is disrupted, it can induce the growth, invasion, and spread of cancer cells. Previous research²² has demonstrated the significant role of miR-340-5p in regulating the growth and spread of laryngeal cancer. Moreover, our independent analysis, which was conducted using Starbase online tools, predicted that miR-340-5p possessed a binding site on Yes-Associated Protein (*YAPI*), one of the genes involved in the Hippo signaling pathway. Nevertheless, there is still a lack of comprehensive understanding regarding the molecular mechanisms through which miR-340-5p controls tumor growth and its spread. The investigation of miR-340-5p expression in head and neck cancer, especially in NPC, and its relevance to the clinicopathological profile and metastasis status of the patients has not been conducted before. Therefore, in this study, we aim to unravel the expression of *YAPI* and miR-340-5p in metastatic nasopharyngeal carcinoma (NPC) cases, exploring a potential correlation in expression between these two markers.

Materials and Methods

Data Collection and Processing

The present study employed the University of California Santa Cruz (UCSC) Xena browser, an internet-based tool for visualizing and analyzing functional genomic data in clinical research²³, to construct a correlation heatmap of the *YAPI* and miR-340-5p genes within a cohort of patients, encompassing both normal and primary tumors using The Cancer Global Atlas Head-Neck Squamous Cell Carcinoma (TCGA HNSC) dataset. The gene and miRNA correlation with the stage of tumor and metastasis status were also investi-

gated using the UCSC Xena browser. In addition, we used StarBase (<http://starBase.sysu.edu.cn/>)^{24,25}, a database for decoding miRNA-ceRNA, miRNA-ncRNA and RNA-protein interaction networks from CLIP-Seq data, to predict potential binding mRNA *YAPI* and miR-340-5p.

Clinical Samples

The medical records and paraffin blocks of Nasopharyngeal Carcinoma (NPC) patients spanning from January 2018 to December 2021 were acquired from the archives of the Anatomical Pathology Department at Cipto Mangunkusumo General Hospital. This study involved 68 tissue specimens from 48 males and 20 females, ranging in age from 19 to 74 years, all with confirmed positive histopathological diagnoses of NPC. To ensure the accuracy of the histopathological classification and the presence of any lympho-vascular invasion, our researchers conducted a thorough re-examination of all collected samples. This study was approved by the Health Research Ethics Committee of the Faculty of Medicine Universitas Indonesia and Dr. Cipto Mangunkusumo National Hospital (HREC FMUICMH) (approval No. 23-05-0633).

Functional Enrichment Analysis

Through data mining in Head and Neck Squamous Cell Carcinoma (TCGA, PanCancer Atlas) using cBioPortal²⁶⁻²⁸ for Cancer Genomics, we identified the most co-upregulated genes with *YAPI* (Spearman's Correlation $r \geq 0.5$). The genes that showed concurrent upregulation with *YAPI* subsequently were loaded into The Database for Annotation Visualization and Integrated Discovery (DAVID)²⁹ to differentiate the function of numerous proteins, then to integrate the information obtained to decipher the Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and REACTOME pathway of identified *YAPI*-associated genes. Functional annotation and enrichment pathways were visualized in the "GOplot" package³⁰.

Protein-Protein Interaction (PPI) Network and Hub-Neighbour Gene Establishment

Search Tool for the Retrieval of Interacting Genes (STRING)³¹ was used to construct protein-protein interaction of co-upregulated genes with *YAPI*. The network was then sent to Cytoscape (San Diego, CA, USA; version 3.10.1)³² software for network visualizing and identifying one hub neighbor of *YAPI*.

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

The RNA isolation process involved homogenizing paraffin block sections, clarifying the supernatant, and purifying RNA using the Filter Spin-Away™ system (Irvine, CA, USA). Ethanol was added to the pass-through, and RNA was eluted for storage. MiRNA extraction utilized RiboEx™ (Daejeon, South Korea), followed by centrifugation, ethanol addition, and purification using mini spin columns. The eluted miRNA was then stored.

For cDNA synthesis, 16 μ l of RNA (equivalent to 1 μ g) was combined with 4 μ l of iScript RT supermix, totaling 20 μ l for the reaction. The RT-PCR protocol comprised initial priming lasting 5 minutes at 25°C, followed by reverse transcription for 30 minutes at 42°C, and subsequent inactivation of reverse transcription for 5 minutes at 85°C. The resulting cDNA was stored at -20°C or subjected to quantitative real-time PCR (qPCR).

In the subsequent RT-qPCR, the amplification of *YAPI* and miR-340-5p utilized specific primers. For *YAPI*: forward, 5'-ACTGCTTCGGCAGGAGTTAG-3'; reverse, 5'-GGTTCGAGGGACACTGTAGC-3'. For miR-340-5p: forward, 5'-GCGGTTATAAAGCAATGAGA-3'; reverse, 5'-GTGCGTGTCGTGGAGTCG-3'. *U6* served as a control with the following primers: forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3'; reverse, 5'-CGCTTCACGAATTTGCGTGCAT-3'.

The expression levels of *YAPI* and miR-340-5p were denoted as cycle threshold (Ct), representing the total fractional cycle when the fluorescence intensity passes a predetermined threshold. Simultaneously, the gene *U6* small nuclear RNA (snRNA) served as an internal control to normalize the relative expression of *YAPI* and miR-340-5p. The normalization process was expressed as delta Ct (Δ Ct), calculated as the disparity between the Ct values of miR-340-5p and *U6* snRNA utilized as the control reference.

Statistical Analysis

The clinicopathological data was presented in the table, and the Chi-square test was used to compare the differences in the clinicopathological profile with the metastasis status. The data expression of mRNA and miRNA were presented in bar charts and analyzed by GraphPad Prism (version 9.5.1 Boston, MA, USA) and SPSS version 29.0.1.0 (IBM Corp., Armonk, NY, USA) software using an independent *t*-test. *p*-value <0.05 was considered to indicate a statistically significant difference. Correlation analysis between *YAPI* and miR-340-5p expression was

Table I. Clinicopathological profile of NPC samples.

Characteristics	Patients N (%)	Metastasis		p-value
		Yes (n=32)	No (n=36)	
Sex				
Male	48 (70.6)	24 (50)	24 (50)	0.627
Female	20 (29.4)	8 (52.9)	12 (47.1)	
Age				
≤50 years	46 (67.6)	25 (54.3)	21 (45.7)	0.138
>50 years	22 (32.4)	7 (31.8)	15 (68.2)	
Smoking history				
Yes	29 (42.6)	13 (44.8)	16 (55.2)	0.369
No	29 (42.6)	16 (55.2)	13 (44.8)	
No data available	10 (14.7)	3 (30)	7 (70)	
Sign and symptoms				
<i>Neck lump</i>				
Yes	56 (82.4)	31 (55.4)	25 (44.6)	0.013
No	11 (16.2)	1 (9.1)	10 (90.9)	
Not available	1 (1.5)	0	1 (100)	
<i>Visual impairment</i>				
Yes	23 (33.8)	10 (43.5)	13 (58.5)	0.565
No	44 (64.7)	22 (50)	22 (50)	
Not available	1 (1.5)	0	1 (100)	
<i>Hearing impairment</i>				
Yes	50 (73.5)	22 (44)	28 (56)	0.369
No	17 (25)	10 (58.8)	7 (41.2)	
Not available	1 (1.5)	0	1 (100)	
<i>Olfactory impairment</i>				
Yes	25 (36.8)	13 (52)	12 (48)	0.599
No	40 (58.8)	17 (42.5)	23 (57.5)	
Not available	3 (4.4)	2 (66.7)	1 (33.3)	
<i>Nerve damage</i>				
Yes	28 (41.2)	15 (53.6)	13 (46.4)	0.465
No	39 (57.4)	17 (43.6)	22 (56.4)	
Not available	1 (1.5)	0	1 (100)	
<i>Pain</i>				
Yes	48 (70.6)	24 (50)	24 (50)	0.542
No	19 (27.9)	8 (42.1)	11 (57.9)	
No data available	1 (1.5)	0	1 (100)	
<i>Bone and joint pain</i>				
Yes	11 (16.2)	9 (81.8)	2 (18.2)	0.009
No	50 (73.5)	18 (36)	32 (64)	
No data available	7 (10.3)	5 (71.4)	2 (28.6)	
T stage				
T1	2 (2.9)	0	2 (100)	0.328
T2	21 (30.9)	9 (42.9)	12 (57.1)	
T3	11 (16.2)	4 (36.4)	7 (63.6)	
T4	34 (50)	19 (55.9)	15 (44.1)	
N stage				
N0	5 (7.4)	0	5 (100)	0.045
N1	10 (14.7)	3 (30)	7 (70)	
N2	22 (32.4)	10 (45.5)	12 (54.5)	
N3	31 (45.6)	19 (61.3)	12 (38.7)	

Table continued

Table 1 (Continued). Clinicopathological profile of NPC samples.

Characteristics	Patients N (%)	Metastasis		p-value
		Yes (n=32)	No (n=36)	
Metastasis target organ				
<i>Bone</i>				
Yes	28 (41.2)			<0.001
No	40 (58.8)			
<i>Liver</i>				
Yes	5 (7.4)			0.026
No	62 (91.2)			
Not available	1 (1.5)			
<i>Lung</i>				
Yes	7 (10.3)			0.007
No	60 (88.2)			
Not available	1 (1.5)			
<i>Brain</i>				
Yes	3 (4.4)			0.081
No	61 (89.7)			
Not available	4 (5.9)			
Histopathological classification				
Keratinizing squamous cell carcinoma	2 (2.9)	2 (100)	0	0.131
Non-keratinizing squamous cell carcinoma	66 (97.1)	30 (45.5)	36 (54.5)	
Lympho-vascular invasion				
Yes	1 (1.5)	1 (100)	0	0.406
No	66 (97.1)	32 (48.5)	34 (51.5)	
Not available	1 (1.5)	0	1 (100)	

performed using Pearson's correlation analysis in NPC metastasis and non-metastasis cases samples.

Results

Clinicopathological Profile of NPC Samples

We first evaluated the clinicopathological features of our samples and their correlation with metastasis status (Table I). The table shows a higher occurrence of NPC in males (70.6%) compared to females (29.4%), but there is no significant difference in metastasis status between males and females ($p=0.672$). NPC was more prevalent among the study participants in individuals aged 50 years or younger (67.6%). A smoking history was reported in 58 patients (85.2%). The predominant symptoms observed in patients were neck lumps (82.4%), which were higher in metastasis cases ($p=0.013$), followed by hearing impairment (73.5%) and pain (70.6%). In particular, bone and joint pains (16.2%) are more common in metastasis cases ($p=0.009$). What

is striking from the table is that NPC patients are most likely to get diagnosed at the T4 stage (50%) and N3 stage (45.6%). Bone metastasis was the most common target organ (41.2%, $p<0.001$), followed by lung (10.3%, $p=0.007$), liver (7.4%, $p=0.026$), and brain (4.4%, 0.081). Non-keratinizing squamous cell carcinoma remains the most frequent subtype of NPC patients (97.1%). Lympho-vascular invasion was found only in one patient (1.5%).

The Expression Level of YAP1 and miR-340-5p in Head and Neck Cancer TCGA Database

According to a recent study¹⁶, *YAP1* has a dual role in cancer depending on the cellular environment, it can act as either an oncoprotein or a tumor suppressor. In this study, we first try to investigate the expression of *YAP1* in a broader sample of head and neck cancer; to verify this, we used data from a TCGA Head and Neck Cancer database, which were analyzed using the UCSC Xena browser (<http://xena.ucsc.edu/>). An overview comparison is presented in Figure 1A. The data showed that *YAP1*

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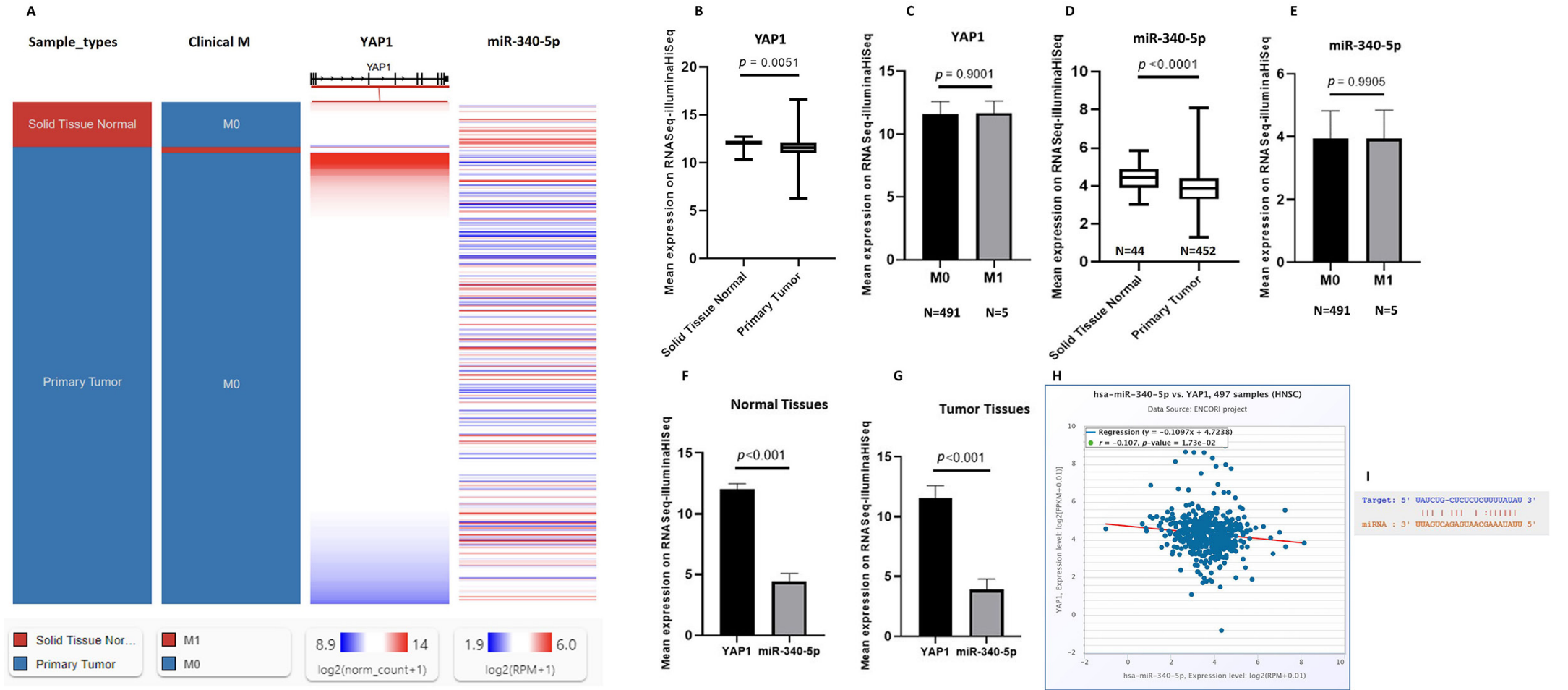


Figure 1. *YAP1* and miR-340-5p expression profile in Head and Neck Cancer TCGA database. **A**, Heatmap of mRNA expression of *YAP1* and miR-340-5p in different sample types and clinical M status. **B-C**, *YAP1* expression in primary tumor and clinical metastasis status. **D-E**, miR-340-5p expression in primary tumor and clinical metastasis status. **F-G**, Comparison *YAP1* and miR-340-5p expression in normal and tumor tissues. **H-I**, Starbase database was used to predict *YAP1* (target) and miR-340-5p correlation and their complementary structure.

was significantly downregulated in tumors compared to normal tissues (Figure 1B), although there was no significant difference between the expression of these markers in clinical metastasis status (Figure 1C).

Some studies³³ have revealed that miRNAs have been implicated in critical NPC processes such as epithelial-to-mesenchymal transition (EMT) and metastasis while further promoting chemoresistance and radioresistance. A prior study²² in laryngeal carcinoma has shown that the expression levels of miR-340-5p were down-regulated, and its overexpression might hamper *in vitro* laryngeal squamous cell carcinoma (LSCC) proliferation and migration. Using the TCGA Head and Neck Cancer database, we discovered a consistent decrease in miR-340-5p expression in head and neck cancer compared to the normal group (Figure 1D), but not significant in metastasis status (Figure 1E). The expression patterns of *YAPI* and miR-340-5p in the head and neck cancer TCGA database were compared, and a clear inverse pattern was identified. In particular, the expression levels of miR-340-5p were notably decreased compared to *YAPI* in both normal and tumor samples (Figure 1F-G). In this study, we also used miRNA target prediction software (Starbase v3.0) to search for the target genes of miR-340-5p. We found that *YAPI* negatively correlates with miR-340-5p (Figure 1H), and there is a predictive structure complementation between *YAPI* as a target of miR-340-5p (Figure 1I).

Functional Enrichment and PPI Analysis of *YAPI* and miR-340-5p Associated Genes

Further analysis was undertaken to elucidate a potential regulatory mechanism by which *YAPI* and miR-340-5p may exert regulatory control in head and neck cancer progression within the broader context. Through data mining in the Head and Neck Cancer cohort in TCGA using cBioPortal for Cancer Genomics, we identified the most co-up-regulated genes with *YAPI* ($r \geq 0.5$) (Supplementary Table I), and subsequently, we constructed PPI network analysis involving *YAPI* (Figure 2A). Within this network, genes directly interacting with *YAPI* were identified as first hub neighbors using Cytoscape. A total of six genes were then analyzed using the DAVID database to perform GO and signaling pathway enrichment (Figure 2B-C).

Terms of the hippo signaling pathway were enriched in the GO biological process. Regarding the GO cellular component, these six genes were main-

ly enriched in bi-cellular tight junction and membrane raft. Turning to the GO molecular function, this gene is mainly enriched in transcription co-activator activity and macromolecular complex binding. Furthermore, the hippo signaling pathway and the *RUNX3* regulates *YAPI*-mediated transcription were both enriched by KEGG and REACTOME pathway analyses, respectively.

In addition, we also presented an analysis of the functional enrichment pathway from the significant target genes of miR-340-5p using miRNA Pathway Dictionary Database (miRPathDB) 2.0³⁴ (Figure 2D-E). The top KEGG pathways of miR-340-5p gene targets were enriched in pathways in cancer, the phosphatidylinositol 3'-kinase-Akt (PI3K-Akt) signaling pathway, proteoglycans in cancer, Rap1 signaling pathway, focal adhesion, endocytosis, MicroRNAs in cancer, and Hippo signaling pathway. The top biological processes associated with miR-340-5p gene targets were transcription by RNA polymerase II, cell cycle, and negative regulation of gene expression. In terms of cellular components, the genes associated with miR-340-5p as a target were enriched in chromosomes. Moreover, looking at molecular functions attributed to these genes, DNA-binding transcription factor activity and RNA polymerase II-specific were the top significant molecular functions.

***YAPI* mRNA and miR-340-5p Expression in Nasopharyngeal Carcinoma Tissue**

Turning now to the experimental validation using a metastasis sample of NPC, the qRT-PCR revealed a notable decrease of *YAPI* mRNA and miR-340-5p expression levels in metastatic samples compared to the non-metastasis group (Figure 3A-B). Having done that, further analysis was performed to dissect the expression patterns of these markers in relation to the pathological staging of the disease. Remarkably, both *YAPI* and miR-340-5p exhibited a significant reduction in expression in stage IVB compared to stage II, III, and IVA groups (Figure 3C-D). Notably, in the metastatic group, miR-340-5p exhibited a slightly higher expression than *YAPI*, albeit not reaching statistical significance (Figure 3E). Conversely, a comparative analysis of *YAPI* and miR-340-5p expressions between the two sample groups demonstrated a statistically significant elevation in *YAPI* expression relative to miR-340-5p in the non-metastatic group (Figure 3F). Despite the negative correlation between the expression levels of *YAPI* and miR-340-5p

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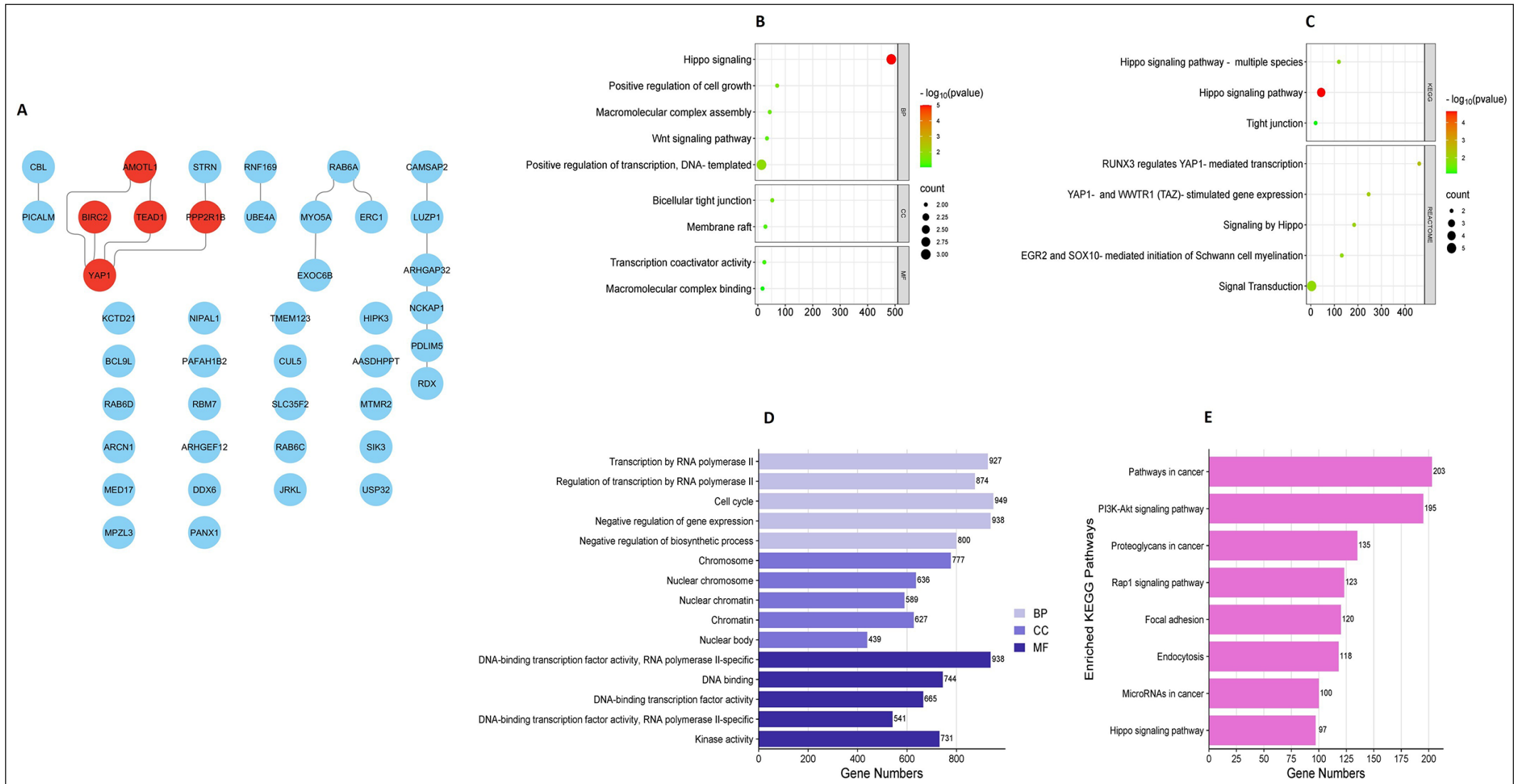


Figure 2. PPI and Functional Enrichment Analysis. **A**, PPI network of *YAP1* and its co-upregulated (Red circle = *YAP1* and one hub neighbor). **B**, Gene ontology of *YAP1* and its one hub neighbor genes. **C**, Enrichment pathway analysis of *YAP1* and its one hub neighbor genes. **D**, Gene ontology of miR-340-5p target genes. **E**, Enrichment pathway analysis of miR-340-5p target genes.

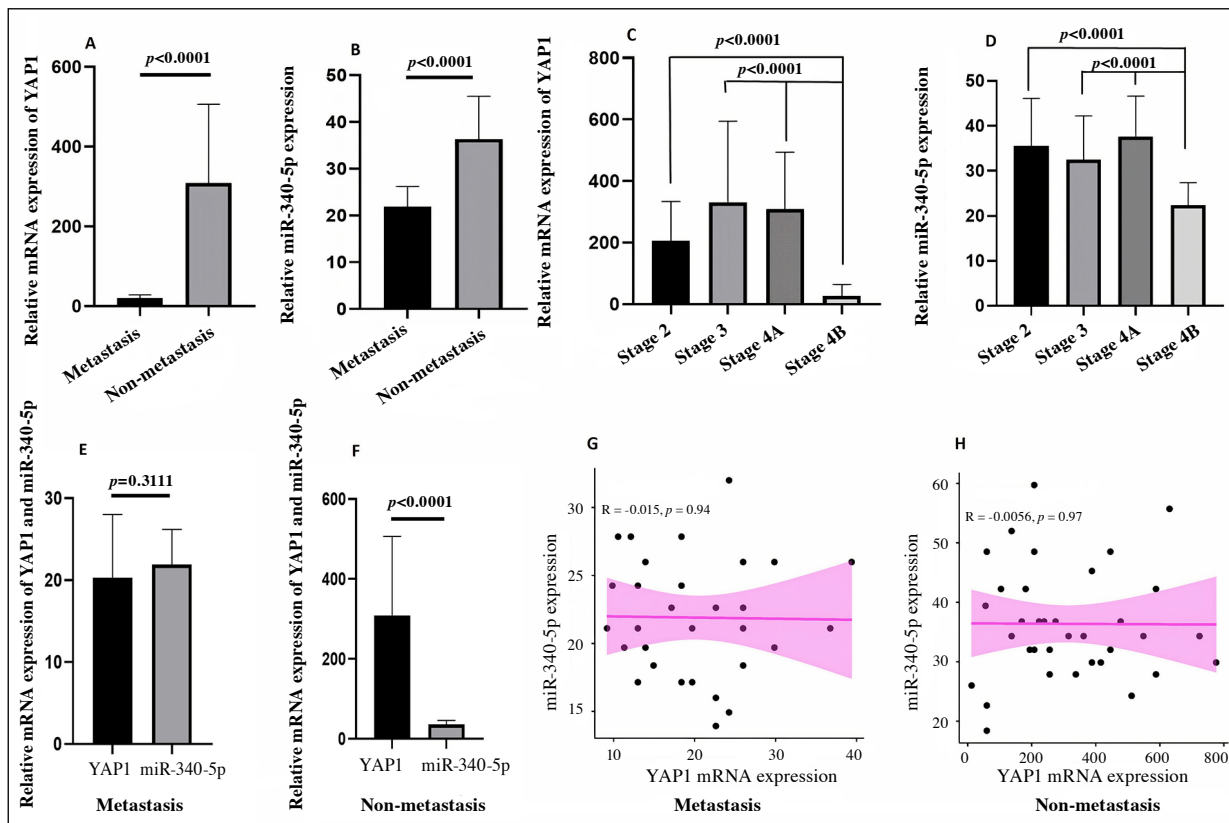


Figure 3. *YAP1* and miR-340-5p are under-expressed in the advanced stage of NPC tissues with metastasis. **A-B**, *YAP1* mRNA and miR-340-5p expression were measured in NPC metastasis and non-metastasis tissues by qRT-PCR. **C-D**, *YAP1* mRNA and miR-340-5p expression were measured in different stages of NPC tissues by qRT-PCR. **E-F**, Comparison of *YAP1* and miR-340-5p expression in metastasis and non-metastasis group, respectively. **G-H**, Pearson's correlation of *YAP1* and miR-340-5p in metastasis and non-metastasis group, respectively.

validated by Pearson's analysis, the statistical significance of this correlation was not achieved (Figure 3G-H).

Discussion

In this study, we analyzed the expression of *YAP1* mRNA and miR-340-5p in NPC samples with and without metastasis to understand their roles in NPC metastasis.

No difference was found in the distribution of metastasis status between males and females. Our study found that most NPC patients were diagnosed at a loco-regionally late stage, particularly T4 (50%), likely due to the nasopharynx's hidden location and nonspecific early symptoms mimicking common upper airway infections^{35,36}. Cervical lymph node enlargement occurred in 82.4% of patients. Apart from cervical lymph node enlargement, there were no significant dif-

ferences in signs and symptoms based on metastasis status. Xu et al³⁷ found that the risk of metastasis increases with higher levels and numbers of involved lymph nodes. Although no significant differences in metastasis status were observed across T and N stages, this study identified that metastasis predominantly occurs in T4 and N3 tumors, aligning with previous research findings^{38,39}. Furthermore, consistent with prior studies³⁹⁻⁴¹, bone metastasis was the most frequent distant metastasis in this study, affecting 41.2% of patients. Previous research⁴² has revealed that the complex interactions between NPC cells and the bone microenvironment, notably through the insulin-like growth factor 1 receptor (IGF-1R), promote tumor proliferation and osteoclast activity, leading to bone metastasis. The study has shown that elevated IGF-1R expression is associated with a higher incidence of bone metastasis, pointing to potential therapeutic targets for intervention.

The study analyzed data from the TCGA Head and Neck Cancer dataset *via* the UCSC Xena browser, revealing a consistent decrease in *YAPI* and miR-340-5p expression levels in tumor tissues compared to normal tissues. Previous study⁴³ on HNSC showed that increased cytoplasmic YAP expression correlates with elevated AKT and YAP phosphorylation, while decreased nuclear YAP expression inversely correlates with these markers. Silencing *YAPI* altered gene expression associated with head and neck squamous cell carcinoma (HNSCC) malignancy, reducing pro-apoptotic genes and increasing survival and angiogenesis-promoting genes⁴³⁻⁴⁶. Overexpression of *YAPI* can promote apoptosis, particularly when localized in the nucleus⁴⁷. *YAPI*'s interaction with *p73* in response to DNA damage has been reported to enhance *p73*-mediated transactivation of apoptotic genes. These cumulative findings underscore the potential tumor-suppressive functions of *YAPI*^{17,48,49}.

Our PPI analysis of *YAPI* and its co-upregulated genes identified several key genes connected to *YAPI*, including angiomin-like 1 (*AMOTL1*), *TEAD1*, protein phosphatase 2 scaffold subunit Abeta (*PPP2R1B*), and baculoviral IAP repeat containing 2 (*BIRC2*). These genes play varied roles in tumorigenesis, with most promoting tumor development⁵⁰⁻⁵³. Notably, *PPP2R1B* also functions as a tumor suppressor⁵⁴. Functional enrichment analysis of these genes highlighted the Hippo signaling pathway as the most enriched KEGG pathway and biological process. Initially discovered in *Drosophila melanogaster* for regulating organ size and cell differentiation, the Hippo pathway is now recognized as crucial in inhibiting tumor growth⁵⁵. Dysregulation of this pathway contributes to tumor invasion, migration, progression, and resistance to cancer therapies, including those for virus-related cancers⁵⁶⁻⁵⁹. Additionally, recent research^{60,61} has identified Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ), downstream effectors of Hippo signaling, as tumor suppressors. Furthermore, this study revealed that "RUNX3 regulates *YAPI*-mediated transcription" as an enriched pathway *via* REACTOME pathway enrichment analysis. Prior studies¹⁹ suggest that YAP1 can act as a tumor suppressor by switching its binding partner to RUNX3 instead of TEAD, demonstrated by reduced tumorigenicity in gastric cancer cells with ectopic RUNX3 expression, dependent on the YAP-RUNX3 interaction.

Our enrichment analysis of miR-340-5p target genes revealed their involvement in several oncogenic pathways, notably the Hippo pathway. Previous studies²² have demonstrated that miR-340-5p suppresses cancer progression by regulating long non-coding RNA nuclear paraspeckle assembly transcript 1 (*NEATI*) and reducing matrix metalloproteinase 11 (*MMP11*) transcription in laryngeal cancer. Lower levels of miR-340-5p are associated with increased tumor size and recurrence in glioblastoma multiforme (GBM)⁶². In esophageal squamous cell carcinoma (OSCC), hypoxic exosomes transfer miR-340-5p to normoxic cells, promoting radio-resistance and a poorer prognosis⁶³.

We validated the expression levels of *YAPI* and miR-340-5p in nasopharyngeal carcinoma (NPC) metastasis tissue samples, finding significantly reduced levels in metastasis tissues compared to primary tumors. Previous studies⁶⁰ have indicated that inhibiting Hippo signaling or activating *YAPI* can markedly reduce the growth of estrogen receptor-positive (ER+) breast cancer cells. At the protein level, YAP expression is significantly lower in breast tumor tissues than in normal tissues, correlating with increased cellular migration when YAP is inhibited¹⁸. Additionally, several explanations also support possible mechanisms suggesting *YAPI* implication as a tumor suppressor gene^{17,19,48,64,65}.

Nevertheless, conflicting data exist. For instance, Huang et al⁶⁶ reported elevated *YAPI* expression in NPC tissues with hepatitis B virus (HBV) infection linked to poor prognosis and EMT pathway regulation. Other studies⁶⁷ have indicated that depleting *YAPI* has been shown to attenuate transforming growth factor beta 1 (TGF- β 1)-induced EMT and concurrently suppress proliferation, migration, and invasion in non-small cell lung cancer (NSCLC). These findings underscore the multifaceted roles of *YAPI* and miR-340-5p in oncogenesis and highlight the need for further research.

It is well-known that microRNAs regulate gene expression by targeting specific sites on transcribed mRNA⁶⁸. Using StarBase^{24,25}, we identified potential complementary sequences of miR-340-5p and *YAPI* and predicted their target sites. Previous studies⁶⁹ have reported that miR-340-5p targets *YAPI* with decreased expression in LSCC, which reduces cell viability and increases apoptosis. This study found significantly lower miR-340-5p expression in metastasis tissue samples, consistent with TCGA Head and Neck Cancer da-

tabase predictions showing lower levels in tumor tissues compared to normal tissues. In this study, miR-340-5p levels correlated with the tumor stage in NPC samples.

Consistent with our findings, several studies^{22,69} have shown decreased miR-340-5p expression in laryngeal cancer tissues and cells, with increased expression reducing growth and cancer spread. Ouet al⁷⁰ reported that miR-340-5p regulates the endoplasmic reticulum stress response in OSCC by targeting protein kinase R (PKR)-like endoplasmic reticulum kinase (*PERK*) and activating transcription factor 6 (*ATF6*), thereby affecting cell proliferation and invasion. Another mechanism by which miR-340-5p suppresses colon cancer migration and invasion is by inhibiting Ras homolog family member A (RhoA) activity, a molecular switch for transmitting signals to the cytoskeleton during cell migration. These findings suggest that miR-340-5p might function as a tumor suppressor^{71,72}.

Regarding other clinicopathological parameters, our findings revealed that advanced-stage tumors (stage 4B) showed significant decreases in *YAPI* and miR-340-5p expression compared to early-stage tumors. Tumor staging is closely correlated with distant metastasis, and larger tumor size is a determinant in SCLC for metastasis occurrence^{73,74}. In colorectal cancer and HNSCC, advanced T stage, histological grade, and positive lymph nodes are linked to higher metastasis likelihood^{75,76}. Our findings align with these correlations, showing the downregulation of *YAPI* and miR-340-5p in advanced NPC stages.

However, this contradicts prior research indicating higher *YAPI* expression in advanced papillary thyroid carcinoma stages⁷⁷. Some studies^{62,78,79} have revealed that lower miR-340-5p levels correlate with advanced tumor stages in various cancers, which is consistent with our results.

Our study characterized *YAPI* and miR-340-5p expression in NPC metastasis tissues, but further research is needed to clarify their mechanistic roles in NPC metastasis. Additional studies with larger samples are needed to understand the diverse functions of *YAPI* and miR-340-5p in cancer growth and metastasis. Our results also provide encouraging directions for future research in this area.

Conclusions

The study set out to characterize the expression of *YAPI* as a downstream of the hippo pathway and miRNA-340-5p in NPC metastasis status. In con-

clusion, our findings shed light on the downregulation of *YAPI* and miR-340-5p using head and neck cancer TCGA database and our NPC metastasis samples, suggesting their potential roles as tumor suppressors. While consistent with previous findings, conflicting reports about the role of *YAPI* and miR-340-5p in tumor progression and metastasis highlight the need for further research.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethics Approval

This study was approved by the Health Research Ethic Committee of the Faculty of Medicine Universitas Indonesia and Dr. Cipto Mangunkusumo National Hospital (HREC FMUICMH) on May 15, 2023 (approval No. 23-05-0633).

Informed Consent

This study is conducted using data collection of stored material (isolate) and thus can be undertaken without direct consent. Any need for direct consent in this study has been waived and approved by the Health Research Ethic Committee - Faculty of Medicine Universitas Indonesia and Dr. Cipto Mangunkusumo National Hospital (HREC FMUI-CMH) in consent waiver statement (No. ND-392/UN2.F1/ETIK/PPM.00.02/2023).

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Authors' Contributions

Lisnawati Rachmadi, Yai Dwina, Marlinda Adham, and Firman Hasan conceptualized the study. Lisnawati Rachmadi, Rahmat Cahyanur, and Firman Hasan developed methodology. Lisnawati Rachmadi, Marlinda Adham, Michelle Linggodigdo, and Rahmat Cahyanur conducted the investigation. Lisnawati Rachmadi, Yai Dwina, Rahmat Cahyanur, Marlinda Adham, Firman Hasan, and Michelle Linggodigdo acquired and interpreted data. Rahmat Cahyanur and Firman Hasan confirmed the authenticity of all the data. Lisnawati Rachmadi, Yai Dwina, Marlinda Adham, Firman Hasan, and Michelle Linggodigdo wrote, reviewed, and edited the manuscript. Lisnawati Rachmadi, Yai Dwina, and Marlinda Adham supervised the study. All authors have read and approved the final version of the manuscript.

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Availability of Data and Materials

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

AI Disclosure

In the production of this study, artificial intelligence (AI) and assisted technologies were utilized to enhance the quality of the manuscript and generate visual data representations. Specifically, the website SRPLOT (<http://www.bioinformatics.com.cn/en>) was used to create some of the figures and visual data representations included in this study. This platform offers bioinformatics tools for producing scientific plots and charts. Additionally, AI tools such as Grammarly were employed to assist in refining the manuscript's language, grammar, and overall readability. This tool was used to ensure clarity and coherence in the presentation of the study's content, supporting the effective and accurate communication of the research findings.

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