Original Article
Clinical significance of AJUBA, YAP1, and MMP14 expression in esophageal squamous cell carcinoma

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Abstract: Esophageal squamous cell carcinoma (ESCC) is generally known to be a highly fatal cancer, and thus novel molecular targets are needed to improve its diagnosis and treatment. AJUBA has been shown to regulate cell cycle, adhesion, proliferation, apoptosis, and migration in many malignant tumors. However, the clinical significance of AJUBA in ESCC tumor metastasis remains unclear. In this study, we explored the role of AJUBA, Yes-associated protein 1 (YAP1), and matrix metalloproteinase 14 (MMP14) in the clinical presentation and survival of ESCC. Immunohistochemical staining showed higher expression of these proteins in cancer tissues than in paired adjacent tissues, and this upregulation was differently related to lymph node metastasis and TNM stage. AJUBA expression was positively correlated with that of YAP1. High expression of MMP14 was associated with reduced survival. In general, our findings reveal that AJUBA, YAP1, and MMP14 might function as oncoproteins and contribute to novel targeted therapy in ESCC.

Keywords: AJUBA, YAP1, MMP14, ESCC, clinical significance

Introduction

Esophageal cancer is one of the most common tumors in the digestive system. Most cases of esophageal cancer derive from esophageal squamous cell carcinoma (ESCC). The advanced stage at initial diagnosis of ESCC results in a poor 5-year overall survival rate of 20.9% [1]. Thus, it is critical to identify the genetic alterations associated with ESCC tumorigenesis or prognosis.

A recent study found that AJUBA, belonging to the LIM domain protein family, could promote tumor invasion and inhibit cisplatin-induced apoptosis in ESCC [2]. The LIM region contains two consecutive zinc finger structures that participate in interactions with multiple proteins and thus contribute to tumor development, with effects on cell mitosis [3], cell adhesion [4], migration [5], transcriptional regulation [6], and immune response [7]. Combined with the serine-threonine kinase aurora A, AJUBA can regulate the cell cycle by promoting mitosis and G2/M phase transition in HeLa cells [8]. AJUBA

can also activate the small GTPase Rac1 to regulate cell adhesion in human keratinocytes [9]. AJUBA interacts with Snail to enhance ability epithelial-mesenchymal transition (EMT) in colorectal cancer cells [10].

Yes-associated protein 1 (YAP1) plays an important role in embryonic development and tumor formation as a crucial downstream effector of the Hippo pathway. It usually acts as an oncogene in gastric cancer [11], bladder cancer [12], and cervical cancer [13]. However, there have been a few studies showing a role of YAP1 in the migration of the neoplasm in ESCC. AJUBA can function as a novel regulator of the Hippo pathway by binding to LATS1/2, thus preventing YAP1 activation in Drosophila [14] and malignant mesothelioma [15]. RNA-seq analysis has shown that AJUBA can regulate the MAPK pathway to activate matrix metalloproteinases (MMP) [16-18]. MMP14 is an extracellular matrix enzyme with the ability to degrade collagen and activate MMP2. In ESCC, overexpression of MMP14 is associated with lymphatic invasion and poor prognosis [19, 20]. Thus,

Table 1. Clinicopathological characteristics of 142 ESCC cases

Characteristics		N (%)
Gender	Male	111 (78.17%)
	Female	31 (21.83%)
Age	≥60	93 (65.49%)
	<60	49 (34.51%)
Pathological grading	Well & Moderate	101 (71.13%)
	Poor	41 (28.87%)
LN metastasis	Present	45 (31.69%)
	Absent	97 (68.31%)
TNM stage	I + II	70 (49.30%)
	III + IV	72 (50.70%)
AJUBA	Positive	124 (87.32%)
	Negative	18 (12.68%)
YAP1	Positive	124 (87.32%)
	Negative	18 (12.68%)
MMP14	Positive	121 (85.21%)
	Negative	21 (14.79%)

Note: LN metastasis is short for lymph node metastasis.

MMP14 also appears to play a crucial role in ESCC.

In the present study, we detected the expression of AJUBA, YAP1, and MMP14 in ESCC using immunohistochemistry (IHC) and explored the relationship between these expression levels and lymph node metastasis. We also conducted a retrospective study to evaluate the role of AJUBA, YAP1, and MMP14 in overall survival. Our results may provide a new molecular target for ESCC therapy and prevention of metastasis.

Materials and methods

Patients and samples

A total of 142 primary ESCC tumor resection samples were collected from the Department of Pathology at the Second Hospital of Hebei Medical University from 2010 to 2015. In addition, the paired adjacent non-cancer samples at least 5 cm from the lesions were also acquired, but 31 of them were removed for failing to get enough squamous epithelium. All of the samples were formalin fixed paraffin-embedded tissues (FFPE). None of the patients underwent preoperative chemotherapy or radiotherapy. Clinical data such as gender, age, tumor differentiation, lymph metastasis, TNM stage (according to the American Joint Com-

mittee on Cancer, 7th edition), and survival were gathered (**Table 1**). We obtained all patients' consent and permission according to the guidelines of the Second Hospital of Hebei Medical University ethics committee.

Immunohistochemistry

Cancer tissues were formalin-fixed and paraffin-embedded into tissue blocks. Then, 4-μm thick paraffin sections were prepared for analysis with a streptavidin-peroxidase (SP) method [21]. After deparaffinization and hydration, antigen retrieval was performed in 0.01 M citrate buffer (pH 6.0) for 10 min. Endogenous peroxidases were quenched with 3% H_2O_2 for 10 min. After treatment with Block Solution (Zhongshanjinqiao, Beijing, China) for 60 min at temperature of 25°C sections were incubated at 4°C overnight with anti-AJUBA (1:300; Cell Signaling Technology, Danvers, MA, USA), anti-YAP (1:400; Abcam, Cambridge, UK), and anti-MMP14 (1:400; Abcam) antibody. An SP IHC Kit (Zhongshanjinqiao) was used for color development with diaminobenzidine tetrahydrochloride. Counterstaining was performed with hematoxylin. Concurrent IHC staining of tissue sections in the absence of the primary antibody was performed as a negative control.

Evaluation of IHC staining results

Two independent pathologists evaluated the IHC staining semi-quantitatively for each case within five randomly selected high-power fields (20× magnification) under a light microscope using the following scoring system: grade 0, <10% immunoreactive cells; grade 1, 10-30% immunoreactive cells; grade 2, 30-50% immunoreactive cells; grade 3, > 50% immunoreactive cells. Cases with grade 0 were regarded as negative, and those with grades 1 to 3 were regarded as positive.

Statistical analysis

Statistical analyses were carried out using SPSS 17.0. Clinicopathological features associated with AJUBA, YAP1, and MMP14 expression were analyzed for statistical significance using the χ^2 or Fisher's exact test. Spearman analysis was used to calculate correlations among factors.

For survival analysis, we underwent a retrospective research. Kaplan-Meier survival cur-
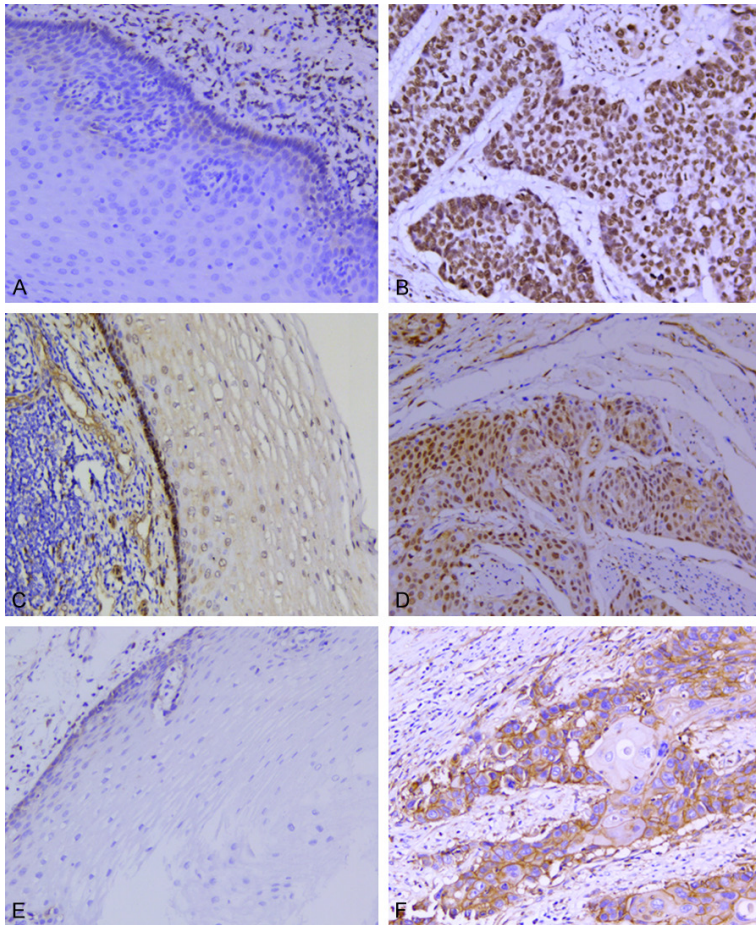
Figure 1. Expression of AJUBA, YAP1, and MMP14 in ESCC and adjacent tissues. IHC staining of AJUBA in the nucleus, YAP1 mainly in the nucleus with some staining in the cytoplasm, and MMP14 in the membrane and cytoplasm. A. No AJUBA expression in adjacent epithelium (×200). B. High AJUBA expression in ESCC (×200). C. No YAP1 expression in adjacent epithelium (×200). D. High YAP1 expression in ESCC (×200). E. No MMP14 expression in adjacent epithelium (×200). F. High MMP14 expression in ESCC (×200).

111 paired adjacent tissues using IHC (**Figure 1**). The results showed that 87.32% (124/142) of tumor tissues exhibited positive expression of AJUBA, whereas 3.60% (4/111) of adjacent tissues were positive for AJUBA. Similarly, 87.32% (124/142) of tumor tissues showed positive expression of YAP1, whereas only 8.11% (9/111) of adjacent tissues did. Finally, 85.21% (121/142) of tumor tissues were positive for MMP14 expression, whereas 5.41% (6/111) of adjacent tissues were positive. These results demonstrated that AJUBA, YAP1, and MMP14 are more highly expressed in tumor than in adjacent tissue samples ($P<0.05$).

AJUBA, YAP1, and MMP14 are associated with tumor invasion and metastasis

Relationships between AJUBA, YAP1, and MMP14 expression and clinicopathological features were further analyzed (**Table 2**). YAP1 and MMP14 were expressed at higher levels in cases with lymph node metastasis than in those without (95.56% vs. 83.51%, $P = 0.045$; 95.56% vs. 80.41%, $P = 0.018$, respectively). Stage III/IV cancer tissues exhibited higher expression of AJUBA and MMP14 than stage I/II tissues (94.44% vs. 80.00%, $P = 0.010$; 91.67% vs. 78.57%, $P = 0.028$, respectively), indicating that AJUBA and MMP14 expression is associated with ESCC tumor malignancy. Taken together, these findings indicate that AJUBA, YAP1, and MMP14 are correlated with tumor invasion and metastasis in ESCC.

AJUBA expression positively correlated with YAP1 but not with MMP14

We analyzed the relationships among AJUBA, YAP1, and MMP14 expression and found that YAP1 expression was positively correlated with AJUBA expression (r = 0.300, $P = 0.001$) and MMP14 expression (r = 0.438, $P = 0.001$),

ves were constructed for groups based on univariate predictors, and differences between groups were assessed with the log-rank test. Within limitations of telephone follow-up, some useful information we failed to get, such as patients' cause of death. So we chose to use overall survival instead of disease-free survival. Multivariate survival analyses were performed using the likelihood ratio test of the Cox proportional hazards model. Differences were assessed with a two-sided test and considered significant at $P<0.05$.

Results

AJUBA, YAP1, and MMP14 proteins are overexpressed in ESCC

We analyzed the expression levels of AJUBA, YAP1, and MMP14 in 142 tumor tissues and

Table 2. Characteristics of AJUBA, YAP1, and MMP14 expression in ESCC tissues

Characteristics	n	AJUBA		P	YAP1		P	MMP14		P
		+	-		+	-		+	-	
Gender										
Male	111	98	13	0.545	98	13	0.513	95	16	0.780
Female	31	26	5		26	5		26	5	
Age										
≥60	93	79	14	0.241	82	11	0.676	82	11	0.171
<60	49	45	4		42	7		39	10	
Pathological grading										
Well-Moderate	101	87	14	0.505	90	11	0.316	88	13	0.312
Poor	41	37	4		34	7		33	8	
LN metastasis										
Absent	97	82	15	0.143	81	16	**0.045**	78	19	**0.018**
Present	45	42	3		43	2		43	2	
TNM stage										
I + II	70	56	14	**0.010**	58	12	0.115	55	15	**0.028**
III + IV[*]	72	68	4		66	6		66	6	

Note: Statistically significant values (P<0.05) are in boldface type. P-values are derived from two-sided tests. [*]For limitation of early medical work, the stage classification was based on clinical information after surgery. Some patients of stage III or IV received surgery.

Table 3. Correlation of ESCC prognosis with AJUBA, YAP1, and MMP14 expression and clinicopathological factors

Characteristics	Univariate		Multivariate[*]		
	χ^2	P	HR	95% CI	P
Gender					
Male vs. Female	0.031	0.860	-	-	-
Age					
≥60 vs. <60	0.152	0.697	-	-	-
Pathological grading					
Poor vs. Well-Moderate	0.583	0.445	-	-	-
LN metastasis					
Present vs. Absent	31.400	**0.001**	1.201	0.665-2.167	0.544
TNM stage					
III + IV vs. I + II	48.266	**0.001**	8.406	3.678-19.209	**0.001**
AJUBA expression					
Positive vs. Negative	2.573	0.109	1.548	0.540-4.437	0.416
YAP1 expression					
Positive vs. Negative	1.104	0.293	0.340	0.117-0.989	**0.048**
MMP14 expression					
Positive vs. Negative	6.598	**0.010**	6.250	1.668-23.412	**0.007**

Note: Statistically significant values (P<0.05) are in boldface type. Log-rank tests were used for univariate analysis, and the enter method was used for multivariate analysis. P-values are derived from two-sided tests. [*]Multivariate test was performed only if the univariate survival test was significant.

while AJUBA expression was not correlated with MMP14 expression (r = 0.020, P = 0.812). This indicates that AJUBA may directly regulate YAP1, which may then regulate MMP14 expression in ESCC.

The prognostic significance of AJUBA, YAP1, and MMP14 expression

To examine whether expression of AJUBA, YAP1, and MMP14 was related to overall sur-
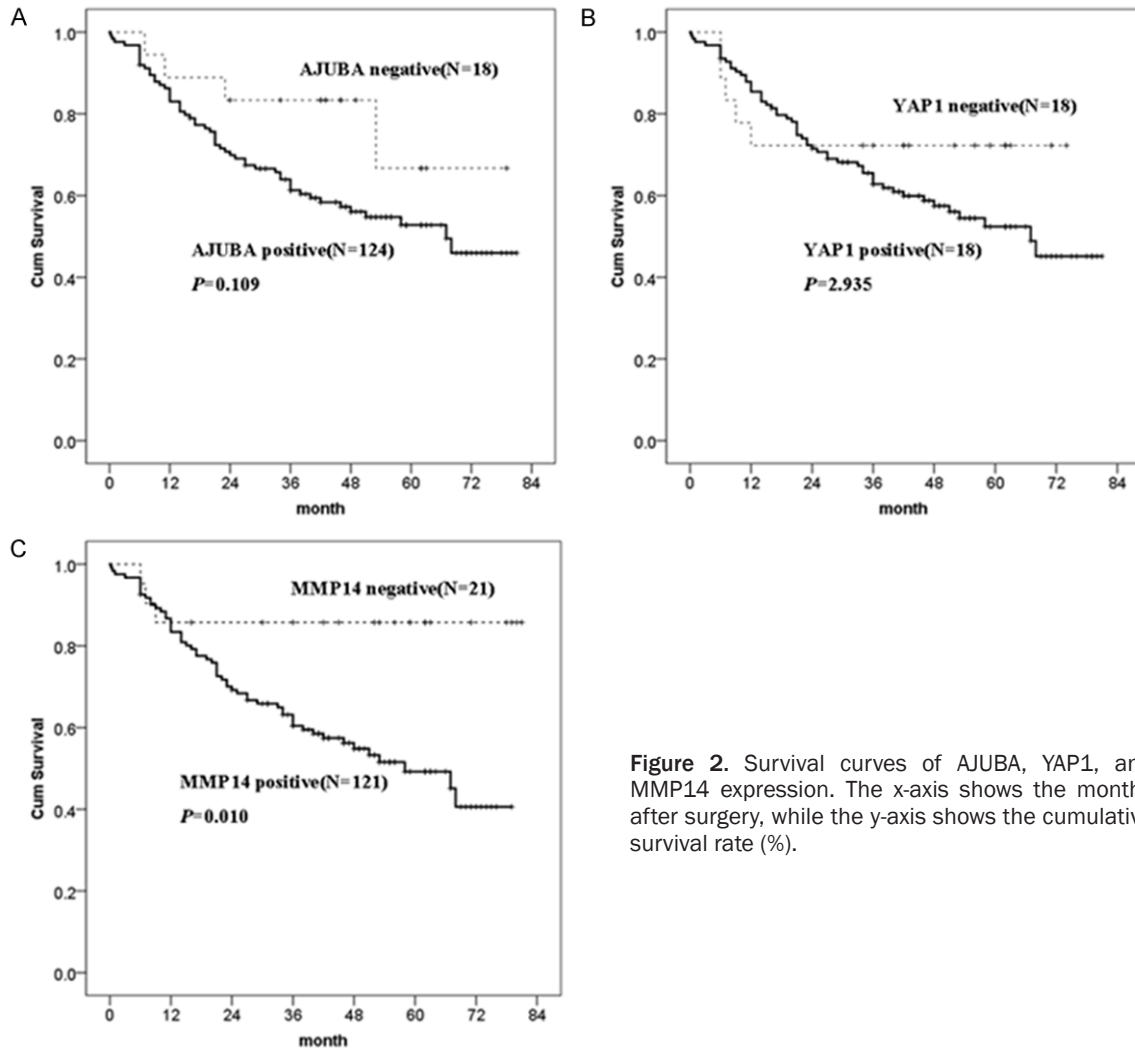
Figure 2. Survival curves of AJUBA, YAP1, and MMP14 expression. The x-axis shows the months after surgery, while the y-axis shows the cumulative survival rate (%).

vival, we performed a Kaplan-Meier analysis and found that lymph node metastasis, stage III/IV, and high expression of MMP14 were associated with reduced survival ($P<0.05$; Table 3; Figure 2). Further, we performed a multivariable Cox regression analysis and found that only MMP14 were independent risk factors of poor overall survival in ESCC (HR = 6.250, 95% CI = 1.668-23.412, P = 0.007; Table 3).

Discussion

AJUBA belongs to the zinc finger protein family in the LIM domain protein family and mediates a wide range of biological functions, including regulation of cell cycle, cell adhesion and ligation, cell migration, cell proliferation and apoptosis, and cell differentiation. AJUBA can inhibit epithelial cell adhesion molecule E-cadherin by recruiting a protein isomer with a 14-3-3 motif

and protein arginine methyltransferase 5 (PRMT5) complex in mouse teratoma cells [22] and human breast cancer cells [23]. In this study, we found that AJUBA expression was elevated in ESCC and related to tumor TNM stage, indicating that it may contribute to tumor invasion in ESCC. Acting as one of the various factors modulating the Hippo pathway, AJUBA can inhibit Hippo pathway signaling by binding to LATS1/2. Hippo signaling is crucial for controlling organ size and proliferation and thus may be involved in tumorigenesis.

Acting as a nuclear effector of the Hippo pathway, YAP1 is overexpressed in numerous tumors, such as oral squamous carcinoma [24], liver cancer [25], and breast cancer [26]. Many studies have focused on the role of YAP1 in tumor apoptosis resistance [27] and metastasis [28]. In this study, we found that expres-

sion of AJUBA was positively correlated with YAP1 expression, indicating the potential for AJUBA to regulate YAP1 in ESCC. Survival analysis showed that high expression of AJUBA and YAP1 was related to poor survival outcomes among ESCC patients. However, whether this phenomenon was related to the binding of AJUBA to LATS1/2 requires further study; we are thus planning future in vitro experiments to explore this molecular mechanism. We also observed that high expression of YAP1 in ESCC tumor tissues was related to lymph node metastasis, indicating that YAP1 may contribute to tumor invasion, consistent with the findings of Pei et al. [29]. Similar findings have been observed in other cancers by Muramatsu et al. [30] and Tanaka et al. [15]. However, Yuan et al. [31] found that YAP1 acted as a tumor suppressor gene in breast cancer, suggesting that the role of YAP1 in various cancers remains controversial. We found that YAP1 served as a protective factor in multivariable Cox regression analysis (HR = 0.341, 95% CI = 0.117-0.989, P = 0.048; **Table 3**).

Next, we assessed MMP14 expression in ESCC and found that elevated expression of MMP14 was related to lymph node metastasis and TNM stage. Bouchard et al. [32] found that MMP14 promoted tumor metastasis in their mouse lung metastasis model. In addition, MMP14 expression was positively correlated with YAP1 expression, indicating that YAP1 may regulate MMP14 expression in ESCC.

In conclusion, our study revealed that AJUBA, YAP1, and MMP14 expression were elevated in ESCC cancer. These proteins may work together to contribute to metastasis in ESCC. MMP14 may also serve as independent risk factors for predicting ESCC patient outcome. Thus, AJUBA, YAP1 and MMP14 may represent novel treatment targets for ESCC.

Disclosure of conflict of interest

None.

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