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Association of low angiomotin-p130 and high YAP1 nuclear expression with adverse prognosis in epithelial ovarian cancer

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Summary. Objectives. The aim of our study was to examine the association of Angiomotin (Amot-p130) and Yes-associated protein 1 (YAP1) expressions and their prognostic significance in epithelial ovarian cancer (EOC).

Methods. A total of 100 primary EOC samples were obtained for immunohistochemical analysis of Amot-p130 and YAP1 expressions. Correlation analysis was performed between Amot-p130 or YAP1 and clinical factors. The overall survival time was calculated.

Results. Low Amot-p130 and high YAP1 nuclear expression were identified in 34 and 56 of 100 EOC tissues, respectively. Both low Amot-p130 and high YAP1 nuclear expression were associated with advanced tumor stage, high-grade carcinoma, and non-response to chemotherapy ($p<0.05$). They were also associated with shorter overall survival time ($p<0.05$) by log-rank test. A marker of low Amot-p130 and high YAP1 expression was associated with high-grade ovarian carcinoma, late-stage disease, non-response to chemotherapy, and shorter overall survival time ($p<0.05$).

Conclusions. Low Amot-p130 and high YAP1 nuclear expression can provide additional prognostic information for patients with EOC. A marker of low Amot-p130 and high YAP1 expression may be a potent predictor of poor prognosis in patients with epithelial ovarian cancer.

Key words: Ovarian cancer, Angiomotin, Yes-associated protein 1, Prognosis

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Introduction

Ovarian cancer (OC) is the third most common gynecological cancer globally, the most lethal gynecological cancer, and the fourth most common cause of cancer-related death in women, with 185,000 deaths occurring annually worldwide (Falzone et al., 2021; Sung et al., 2021). Epithelial ovarian cancer (EOC) is an age-related disease and is mainly considered a postmenopausal disease (between 55-65 years old). EOC comprises approximately 90% of OC, and 70% of EOC patients were diagnosed with end-stage disease with a five-year survival rate of less than 40% (Mohammadian et al., 2017; Momenimovahed et al., 2019). The relationship between age and the outcome of ovarian cancer is uncertain. However, many expressed that younger women with OC are associated with a good prognosis, and an older age is related to a more progressed illness and lower survival rate. Because of the high rate of recurrence and resistance to chemotherapy, the long-term prognosis of EOC patients is poor, with a five-year survival rate of around 30%, especially in advanced-stage disease (Kipps et al., 2013; Siegel et al., 2014). Therefore, there is a need for an increased understanding of signaling mechanisms that drive ovarian tumor behaviors that can be translated into novel therapeutic intervention approaches.

Angiomotin (Amot) was initially identified as an angiostatin-binding protein and characterized by conserved coiled-coil domains and C-terminal PDZ-binding motifs, regulating endothelial cell migration, and tube formation (Troyanovsky et al., 2001; Aase et al., 2007; Dai et al., 2013; Leung and Zernicka-Goetz, 2013). The Amot family consists of three members: Amot (p80 and p130 isoforms), Amot-like protein 1 (AmotL1), and Amot-like protein 2 (AmotL2) (Bratt et al., 2002; Moleirinho et al., 2014). There is a high degree of similarity between the Motin proteins, however, there are functional differences that are still not

fully understood. The two AMOT isoforms, p80 and p130, are only distinguished by the absence or presence of an extended N-terminal domain and exert different effects on cancer cell proliferation (Ernkvist et al., 2006; Ranahan et al., 2011; Adler et al., 2013). Amot-p80 is commonly accepted to play a role in the promotion of endothelial cell migration and angiogenesis, whereas the role of Amot-p130 in cancer is controversial. Yes-associated protein (YAP) is a human oncogene and a potent promoter of organ size, which is a key downstream effector of the Hippo pathway. In cell line experiments, Amot-p130 showed the ability to inhibit YAP1, indicating its potential as a tumor suppressor (Paramasivam et al., 2011; Zhao et al., 2011; Hsu et al., 2015). However, some studies indicated that Amot-p130 prevents Yap phosphorylation and facilitates tumorigenesis, suggesting that Amot is actually an oncogene that can regulate many tumor cell processes (Yi et al., 2013; Lv et al., 2016).

There is conflicting evidence and a growing debate about the function of Amot-p130 in various tumors. Whether *in vitro* or *in vivo*, investigations assessing the role of Amot-p130 in the regulation of OC were poor. In addition, the association of the combined marker of Amot-p130 and YAP1 expression with clinical and pathological characteristics remains uncertain. Therefore, we conducted this study to investigate the expression profiles of Amot-p130 and YAP1 in clinical samples of human EOC tissue and assessed their potential associations with patients' clinical and pathological data, as well as overall survival (OS).

Materials and methods

Patients and clinicopathologic data

A total of one hundred primary EOC samples were obtained from the Xingtai People's Hospital between January 2008 and December 2020. The ovarian cases included 60 serous carcinomas (Fig. 1A), 30 mucinous carcinomas (Fig. 1B), and 10 endometrioid carcinomas (Fig. 1C). Meanwhile, tissues from 20 normal ovaries and 30 benign ovarian neoplasms were collected as controls. The staging surgery was performed in OC patients and all patients were treated with platinum-based chemotherapy after surgery; none of them had any prior history of chemoradiation, radiation, or hormonal therapy before surgery. Clinical and pathological data of patients, such as age, histologic type, tumor grade, tumor stage, lymph node metastases, and response to chemotherapy, were collected in this study. OS was calculated from the time of the first surgery to the date of death or the last follow-up date. Informed consent was obtained from each participant. The study was conducted in accordance with the Declaration of Helsinki.

The histopathologic subtype of ovarian tumor was determined following the World Health Organization criteria and the classification of tumor grade was performed based on a two-tier system (low-grade and high-grade) (Malpica et al., 2004). Histological staging for ovarian tumors was according to the International Federation of Gynecology and Obstetrics (FIGO) staging criteria (Shepherd, 1989). For response to chemotherapy, patients were defined as responders who had progressive or recurrent disease longer than 6 months after withdrawing the first chemotherapy or non-responders who had progressive or recurrent disease after a treatment-free interval of primary platinum-based chemotherapy <6 months (Alberts et al., 2004).

Immunohistochemistry

Formalin-fixed, and paraffin-embedded blocks were retrieved from pathological storage and were freshly cut (4 μm). The 4-μm-thick sections were dried at 68ºC for 2 hours, dewaxed, rehydrated conventionally, and subjected to heat-induced antigen retrieval with 0.01 mol/l citrate buffer for 10 min. Afterward, slides were incubated with 3% H_2O_2 to block endogenous peroxidase, and then with 20% normal goat serum for 30 min at room temperature to reduce nonspecific binding. Next, slides were incubated overnight at 4°C with primary antibodies specific for Amot-p130 (ab85143, 1:100, Abcam) and YAP1 (ab56701, 1:100, Abcam), and then incubated with biotin-labeled anti-rabbit (mouse) IgG antibody for 20 min. Diamino-benzidine was used for visualizing the reactions. Finally, slides were counterstained with hematoxylin. Negative controls were made by replacing the primary antibody with phosphate-

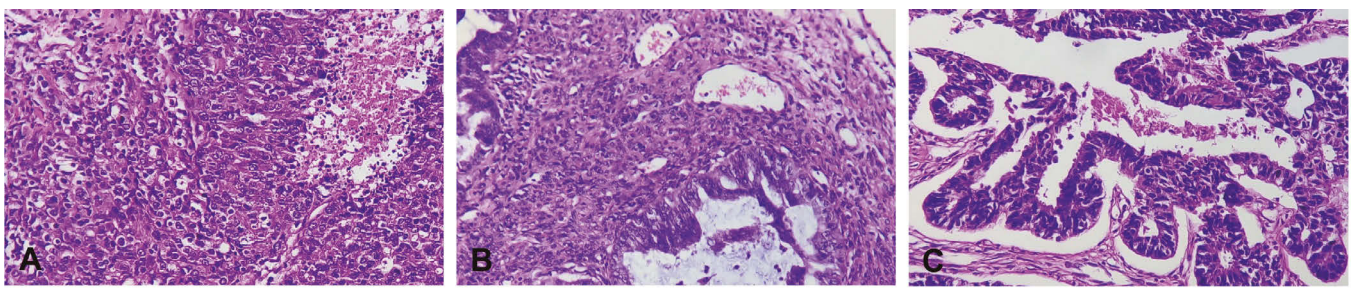

Fig. 1. Hematoxylin-eosin staining of ovarian carcinoma tissues. **A.** Serous carcinomas. **B.** Mucinous carcinomas. **C.** Endometrioid carcinomas. × 200.

buffered saline.

Amot-p130 and YAP1 expression levels were independently evaluated by two pathologists blinded to the clinicopathological patient data. The cytoplasmic staining of Amot-p130 was defined as positive. The nuclear and cytoplasmic staining of YAP1 were analyzed separately. As previously described, the immunostaining of Amot-p130 and YAP1 in tumor samples was semi-quantitatively evaluated (El Hafez and El-Hadaad, 2014). Staining intensity was scored as 0 for no staining; 1 for weak staining; 2 for moderate staining; or 3 for strong staining. The extent of staining was scored according to the percentage of positively stained cells as follows: 0 (≤5%), 1 (6-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%). The average number of positively stained cells was determined by counting cells from 10 random fields at x400 magnification. The final immunohistochemical staining score was obtained by multiplying the staining intensity and the extent of staining (negative expression, scores 0-2; weak expression, scores 3-5; moderate expression, scores 6-9; and strong expression, scores 10-12). For statistical purposes, we classified the negative, weak, and moderate expression cases as a group with reduced expression of Amot-p130, and samples with >50% of positively stained nuclei or cytoplasm with moderate to strong intensity in tumor cells were categorized as high nuclear or cytoplasmic YAP1 expression (Paramasivam et al., 2011).

Statistical analysis

The chi-square test or Fisher's exact test was performed to evaluate the association between clinicopathologic variables and immunohistochemical profiles of Amot-p130 and YAP1. OS was estimated by the Kaplan-Meier method, and the significance of differences in the cumulative survival curves was evaluated by the log-rank test. A significant difference was defined as $p<0.05$. All statistical analyses were performed using SPSS (version 19.0; IBM SPSS, Chicago, IL, USA).

Results

Clinical and pathological characteristics

As shown in (Table 1), this study consisted of 36 patients aged ≤50 years and 64 patients aged >50 years. Approximately 57% of patients were diagnosed with lymphatic metastasis. The majority of patients (68%) presented with FIGO stages III and IV, and a high percentage of carcinomas were of high tumor grade (37%). Carcinomas with a serous histology were the most frequent (60%), whereas mucinous tumors were less common (30%). The mucious tumors with expansive patterns comprised 16 cases and with infiltrative patterns 14 cases. The low-grade endometrioid tumors comprised six cases, with a high grade in four cases. The median survival time of the patients was 40.86 months (range 7-80).

The results of the immunohistochemistry indicated that Amot-p130 expression occurred in the cytoplasm. For the positive control, non-tumorous ovarian tissues displayed a diffuse and strong immunoreactivity for Amot-p130. Among EOC cases, 34 displayed low expression of Amot-p130 (Fig. 2A), while high Amot-p130 expression was identified in 66 cases (Fig. 2B).

Table 1. Association of AMOT-p130 or YAP1 nuclear expression with the clinicopathologic variables of primary EOC patients.

	Total number	Amot-p130				YAP1			
		High (%) n=66	Low (%) n=34	χ^2	*p*	High (%) n=48	Low (%) n=52	χ^2	*p*
Age (years)				3.477	0.062			0.514	0.473
≤50	36	28 (78)	8 (22)			19 (53)	17 (45)		
>50	64	38 (59)	26 (41)			29 (45)	35 (55)		
Histology				3.743	0.154			3.245	0.197
Serous	60	44 (73)	16 (27)			33 (55)	27 (45)		
Mucinous	30	16 (53)	14 (47)			12 (40)	18 (60)		
Endometrioid	10	6 (60)	4 (40)			3 (30)	7 (70)		
Tumor stage				9.694	0.002			7.448	0.006
I-II	32	28 (88)	4 (12)			9 (28)	23 (72)		
III-IV	68	38 (56)	30 (44)			39 (57)	29 (43)		
Tumor grade				51.544	0.000			25.750	0.000
Low-grade	63	58 (92)	5 (8)			18 (29)	45 (71)		
High-grade	37	8 (22)	29 (78)			30 (81)	7 (19)		
Lymph node metastases				2.077	0.150			0.021	0.884
Absent	43	25 (58)	18 (42)			21 (49)	22 (51)		
Present	57	41 (72)	16 (28)			27 (47)	30 (53)		
Response to chemotherapy				9.265	0.002			18.705	0.000
Response	67	51 (76)	16 (24)			22 (33)	45 (67)		
Non-response	33	15 (45)	18 (55)			26 (79)	7 (21)		

Low Amot-p130 expression was detected in 16 serous tumors, 14 mucinous tumors, and 4 endometrioid tumors. In benign ovarian tumor tissues and normal ovarian tissues, Amot-p130 was diffusely positively stained (22/30 and 17/20, respectively). Forty-eight EOC cases showed high nuclear expression of YAP1 (Fig. 2C), and low expression was found in 52 cases (Fig. 2D). High nuclear expression of YAP1 was detected in 33 serous tumors, 12 mucinous tumors, and 3 endometrioid tumors. High YAP1 cytoplasmic expression was observed in 51 EOC cases. In contrast, YAP1 showed only weak positive staining in 17 out of 30 (56.7%) benign ovarian tumor tissues, and normal ovarian tissue was only weakly positive for YAP1 (3/20, 15%).

YAP1 cytoplasmic expression was not related to any clinicopathological findings, and neither were Amot-p130 expression (p=0.736) or YAP1 nuclear expression (p=0.589). The relationships between Amot-p130 and YAP1 nuclear expression and clinicopathological parameters are shown in Table 1. Low expression of Amot-p130 and high nuclear YAP1 expression were correlated with tumor grade (p<0.001), tumor stage (p=0.002 and p=0.006, respectively), and response to chemotherapy (p=0.002 and p<0.001, respectively). Low Amot-p130 expression was significantly associated with high nuclear YAP1 expression in EOC tissues (p=0.016) (Table 2).

To explore potential correlation factors associated with a combined marker of Amot-p130 and YAP1 nuclear expression, patients were divided into four groups: low Amot-p130 with high YAP1 expression, low Amot-p130 with low YAP1, high Amot-p130 with high YAP1, and high Amot-p130 with low YAP1. As shown in Table 3, low Amot-p130 with high nuclear YAP1 expression always co-occurred in OC tissues from patients with late-stage disease (p=0.042), high-grade OC (p<0.001), and non-response to chemotherapy (p<0.001)

Kaplan-Meier analysis showed that EOC patients with low Amot-p130 expression exhibited a significantly reduced OS (p<0.001, Fig. 3) than those with high expression. In addition, patients who had OC with high

Table 2. Association between AMOT-p130 and YAP1 nuclear expression Amot-p130.

	Amot-p130		
	Low (%)	High (%)	*p*-value
YAP1 nuclear			
Low(%)	12 (27)	40 (61)	0.016
High(%)	22 (73)	26 (39)	

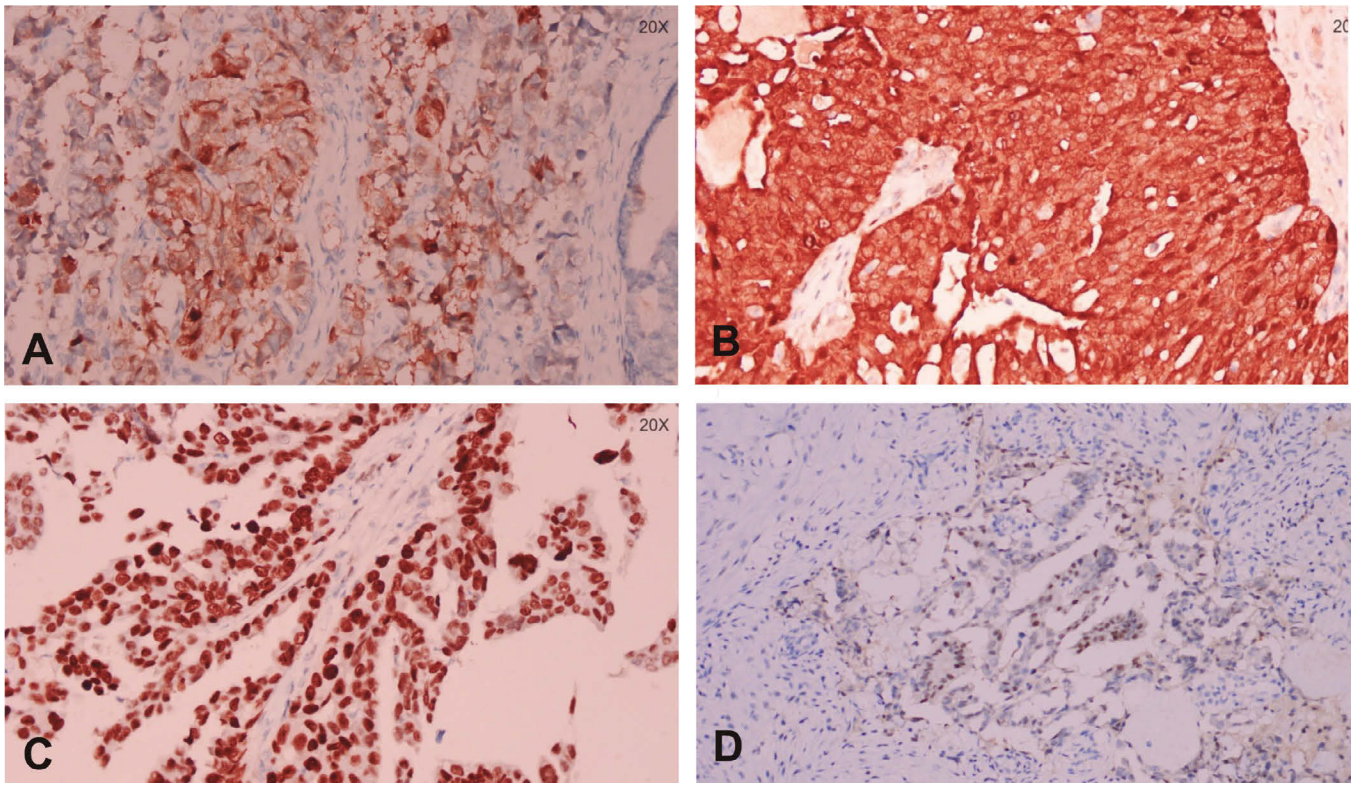

Fig. 2. Representative images of Amot-p130 and YAP1 immunoreactivity in ovarian carcinoma tissues. **A.** Low Amot-p130. **B.** High Amot-p130. **C.** High nuclear YAP1. **D.** Low nulear YAP1. × 200.

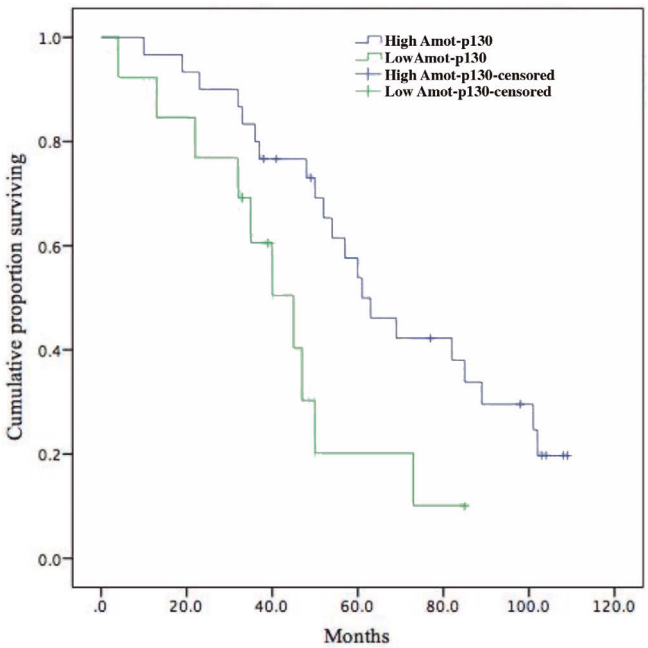

Fig. 3. Survival analysis of EOC patients with low (green line) and high (blue line) expressions of Amot-p130.

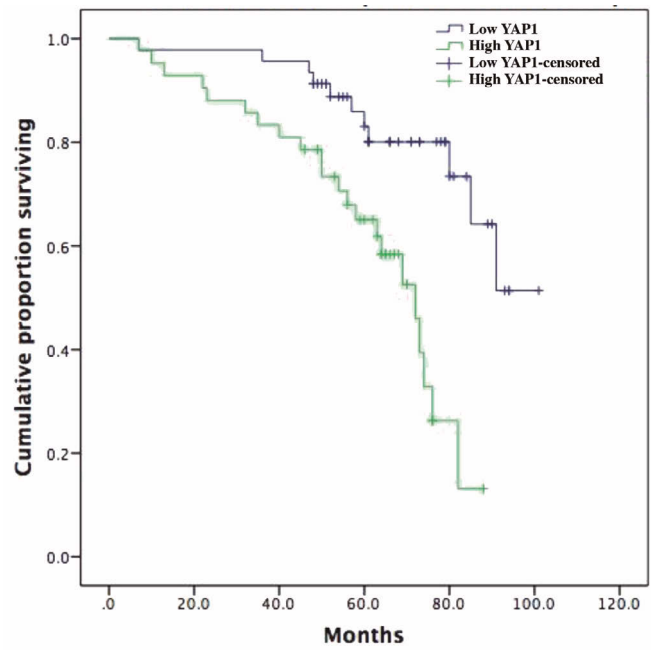

Fig. 4. Survival analysis of EOC patients with high green line) and low (blue line) expressions of YAP1.

Table 3. Association between AMOT-p130 combined with YAP1 nuclear expression and clinicopathologic variables of primary EOC patients.

Variable	Low Amot-p130/ High YAP1 (n=22) 18	Low Amot-p130/ Low YAP1 (n=12) 8	High Amot-p130/ High YAP1 (n=26) 29	High Amot-p130/ Low YAP1 (n=40) 45	Total number	χ^2	*p*
Age (years)							0.201
≤50	5	3	9	19	36	4.630	
>50	17	9	17	21	64		
Histology							
Serous	13	6	16	25	60		0.096
Mucinous	6	4	8	12	30	1.499	
Endometrioid	3	2	2	3	10		
Tumor stage							0.042
I-II	2	5	12	13	32	8.220	
III-IV	20	7	14	27	68		
Tumor grade							0.000
Low-grade	5	8	20	30	63	20.010	
High-grade	17	4	6	10	37		
Lymph node metastases							0.957
Absent	10	5	12	16	43	0.315	
Present	12	7	14	24	57		
Response to chemotherapy							0.005
Response	9	9	23	25	66	12.669	
Non-response	13	3	3	15	34		

Kaplan-Meier analysis showed that EOC patients with low Amot-p130 expression exhibited a significantly reduced OS ($p<0.001$, Fig. 3) than those with high expression. In addition, patients who had OC with high nuclear YAP1 expression had a lower OS rate than those with low nuclear YAP1 expression ($p<0.001$, Fig. 4). However, there was no significant difference in OS ($p=0.15$) between patients with high and low cytoplasmic YAP1 expression.

nuclear YAP1 expression had a lower OS rate than those with low nuclear YAP1 expression (p<0.001, Fig. 4). However, there was no significant difference in OS (p=0.15) between patients with high and low cytoplasmic YAP1 expression.

Discussion

Based on the results of our study, both low Amot-p130 and high nuclear YAP1 expression were found to be potent prognostic factors of poor clinical outcomes in patients with EOC. The expression of the two proteins was strongly associated with tumor stage, tumor grade, and response to chemotherapy. In addition, patients with low Amot-p130 and high nuclear YAP1 expression always had shorter OS times. Moreover, we also identified the effects of a combined marker of Amot-p130 and YAP1 expression on patients' OS. Patients with not only low Amot-p130 but also high nuclear YAP1 expression had the shortest median OS of the four groups. However, YAP1 cytoplasmic expression was not related to any clinicopathological findings or significant differences in OS.

The Amot family plays an important role in tube formation, migration of endothelial cells, the regulation of tight junctions, polarity, and epithelial-mesenchymal transition (EMT) in epithelial cells. Moreover, these proteins regulate the proliferation and migration of cancer cells. However, whether Amot has tumorigenic properties or tumor suppressive functions has been poorly understood in various cancer types to date. In this study, we examined the expression levels of Amot-p130 in EOC tissues via immunohistochemical staining. Our patients exhibited high expression of Amot-p130 in non-tumorous ovarian tissues and benign ovarian tumors but low expression in partial EOC. Therefore, we could hypothesize the possibility of Amot-p130 having an anti-tumorigenic property in the ovary. Consistent with our study, the overexpression of Amot-p130 inhibits growth and soft agar colony formation of OC cells *in vitro*, and the knockdown of Amot-p130 in lung cancer cell lines promotes cancer proliferation, migration, invasion, and EMT, supporting that Amot-p130 may act as a tumor suppressor in tumorigenesis (Hsu et al., 2015; Wang et al., 2017).

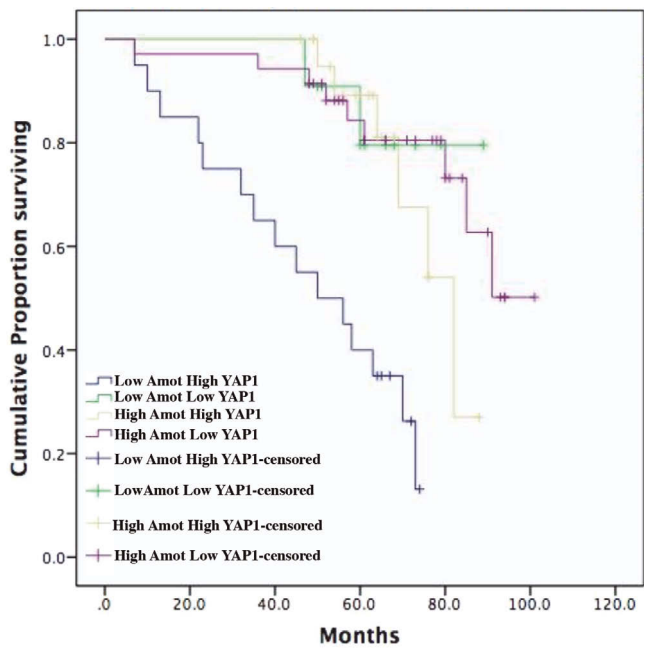

Fig. 5. Survival analysis of EOC patients with (low) or without (high) Amot-p130 in addition to with (high) or without (low) YAP1 expression.

With a cytogenetic location at chromosome Xq23, the Amot gene encodes two different isoform proteins, the so-called p80 (675 amino-acid protein) and p130 (1084 amino-acid protein) (Troyanovsky et al., 2001; Ernkvist et al., 2006). Amot-p130 carries an extended N-terminal of 409 amino acids and this is the only structural difference from Amot-p80 (Ernkvist et al., 2006). Amot-p80 promotes, whereas Amot-p130 suppresses, cancer cell proliferation in breast cancer cell lines (Ranahan et al., 2011; Adler et al., 2013). In addition, Amot-p130 could interact with the Hippo pathway whereas Amot-p80 does not (Moleirinho et al., 2014). Lv et al. (2015) reported that frequent expression of Amot in breast cancer tissue was greater than in non-cancerous breast tissues, implying a potential oncogenic role in tumorigenesis. However, as described by the authors, the Amot antibody they used could detect both isoforms (Amot-p80 and p130), whereas our antibody was specific anti-p130, enabling us to evaluate the exact role of this protein in EOC. Our study demonstrated that low Amot-p130 expression was significantly associated with advanced tumor stage, high-grade, and non-response to chemotherapy, suggesting that the reduction of Amot-p130 expression levels has a close relationship with poor clinical outcomes of patients with OC.

The Hippo-YAP pathway plays a central role in various cellular behaviors, including proliferation, survival, and cell contact inhibition (Zhao et al., 2007; Zeng and Hong, 2008). This pathway is also altered and implicated as an oncogene in a variety of human cancers, including EOC. As an oncogene and the major downstream effector of the Hippo pathway, YAP1 amplification and overexpression were observed in various human cancers and related to the malignant potential of gastric carcinoma, hepatocellular carcinoma, lung cancer, and ovarian cancer (Da et al., 2009; Wang et al., 2010; Zhang et al., 2011; Harvey et al., 2013). In the present study, we found weakly positive YAP1 staining in benign ovarian tumor tissues and normal ovarian tissue. In contrast, there was strong and diffuse nuclear and cytoplasmic YAP1 expression in EOC tissues. Especially, high nuclear YAP1 expression was significantly associated with high-grade ovarian carcinoma, late-stage disease, and non-response to chemotherapy, indicating a poor prognosis in EOC patients. The results were consistent with some earlier

studies (Fu et al., 2014; ; Xia et al., 2014; He et al., 2015). Notably, Hong et al. (Hong et al., 2017) reported that their data revealed no significant association of YAP1 expression with prognosis in gastric cancer. This discrepancy may be attributable to the heterogeneity of the different tumors.

Recent studies have shed light on the role of Angiomotins and other members of the Motin protein family in epithelial cells and pathways directly linked to the pathogenesis of cancer. In particular, Motins have been shown to play a role in signaling pathways regulated by the Hippo-YAP pathway. In our study, we explored the association of combined markers including Amot-p130 and YAP1 with patients' clinical and pathological characteristics, as well as OS times. Patients with low Amot-p130 expression combined with the high YAP1 nuclear expression group exhibited high-grade cancer, late-stage disease, and non-response to chemotherapy. Accordingly, patients in this group had the shortest median OS between the four groups. Consistent with the results of our study, a combined role of Amot-p130 and YAP1 in carcinogenesis has been confirmed in lung, breast, and gastric cancer (Adler et al., 2013; Hsu et al., 2015; Hong et al., 2017). As described in a previous study, Amot-p130 promotes YAP phosphorylation and inhibits the oncogenic activity of YAP in MDCK cells, indicating a potential tumor suppressor function of Amot-p130 (Zhao et al., 2011). However, the reliability of these findings is limited by the small number of cases (n=22). Further validation studies with a larger number of cases will be required to validate the role of Amot-p130 in EOC.

YAP and TAZ are transcription co-activators and major downstream targets regulated by the Hippo pathway (Park and Guan, 2013). Amot-p130 can regulate the Hippo pathway by sequestrating YAP/TAZ in the cytoplasm and increasing the activity of tumor-suppressor LATS1/2, resulting in YAP/TAZ degradation (Chan et al., 2011; Paramasivam et al., 2011; Adler et al., 2013). The Amot stabilizing agent, Tankyrase inhibitor, can cause acceleration of YAP protein degradation, leading to a decrease in the invasiveness of breast cancer cell lines (Wang et al., 2015). Thus, AMOT could be an attractive therapeutic target in EOC, and an evaluation of AMOT expression could be helpful in developing YAP-targeting cancer treatment as well as appropriate patient selection.

Conclusions

In conclusion, we have determined the role of low Amot-p130 expression coupled with high YAP1 expression in 100 patients with EOC. In our study, the expression patterns of the two proteins in EOC tended to be related to unfavorable clinicopathological factors and serve as an independent prognostic factor. They also seem to be an independent predictive factor of recurrence, nevertheless more cases are needed to validate this. Additional studies are required to identify the exact mechanism of Amot-p130 downregulation in EOC. Cases of EOC displaying an expression profile of low Amot-p130 and high YAP1 expression may represent a potential target for tankyrase inhibitor treatment in the future.

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Availability of data and materials. The materials and data generated and/or analyzed in this study are available from the corresponding author upon reasonable request.

Authors' contributions. BC Su and CH Ting performed the experiments. KY Lee, SM Wu, PH Feng, and YF Chan designed the study. BC-Su, CH-Ting, and JY Chen wrote the manuscript.

Ethics approval and consent to participate. The use of human tissue specimens was approved by the Institutional Review Board at Shuang Ho Hospital, Taipei Medical University (IRB No: N201702026). This study was conducted in accordance with the Declaration of Helsinki.

Patient consent for publication. Not applicable.

Competing interests. The authors have no competing financial interests to declare.

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