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Angiopoietin-1 is associated with a decreased risk of lymph node metastasis in early stage cervical cancer

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Summary. Objectives. Lymph node metastasis (LNM) is an important determinant of prognosis in patients with cervical cancer. Members of the angiopoietin family have been demonstrated to regulate tumor-associated angiogenesis and lymphangiogenesis. This study aimed to investigate the expression levels of angiopoietin-1 (ANG1) and angiopoietin-2 (ANG2) in clinically early stage of cervical cancer along with their correlations with LNM.

Methods. In total, 124 human cervical cancer cases classified into stage IA-IIB in accordance with the International Federation of Gynecology and Obstetrics (FIGO) 2009 staging criteria were included. ANG1 and ANG2 expression levels in the tumor sections were assessed by immunohistochemistry (IHC). Univariate and multivariate logistic regression models, including age at diagnosis, FIGO stage, tumor size, pathological type, histological grading, depth of stromal invasion, lymph-vascular space invasion (LVSI) and the expression status of ANG1 and ANG2, were used to evaluate the odds ratios (ORs) for LNM.

Results. ANG1 and ANG2 were positively expressed in 75 (60.5%) and 89 (71.8%) cervical cancers respectively, with predominant staining in the cytoplasm. ANG1 expression was significantly decreased in tumors with LNM, while no correlation was observed between ANG2 expression and LNM. More importantly, the multivariate logistic regression analysis demonstrated that high ANG1 expression was an independent protective factor of LNM (OR 0.107, 95% confidential interval [CI] 0.020~0.567), while LVSI was an independent risk factor of LNM (OR 34.313, 95% CI 5.914~199.092).

Conclusion. ANG1 is associated with a significantly decreased risk of LNM in early stage cervical cancer. The predictive value and role of ANG1 in LNM needs to be further investigated in future studies.

Key words: Angiopoietin-1, Angiopoietin-2, Lymph node metastasis, Cervical cancer

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Introduction

Cervical cancer ranks fourth for both incidence and mortality in women worldwide (Bray et al., 2018), with approximately 90% of cases occurring in low- and middle-income countries or areas (Cohen et al., 2019). Patients with lymph node metastasis (LNM) exhibit a significantly poorer survival compared to those that do not have LNM (Ho et al., 2004; Atahan et al., 2007; Ferlay et al., 2013). Thus, an intensive therapy and surveillance program should be considered in these patients who are at high-risk. In early stage cervical cancer, the incidence of LNM is up to a quarter (Matsuo et al., 2013, 2020). However, the preoperative evaluation of LNM remains challenging despite the advances in the

imaging tests (Bellomi et al., 2005; Bhatla et al., 2019). Hence, the identification of biomarkers for predicting LNM in early stage cervical cancer might improve the personalized treatment for patients.

Angiopoietins (ANGs) including ANG1-4, are a family of secreted glycoproteins acting primarily on the vasculature to regulate blood and lymphatic vessel remodeling and stability (Weis and Cheresh, 2011; Saharinen et al., 2017). As the most crucial members of angiopoietins in human, ANG1 and ANG2 exert various effects under physiological and pathological conditions by binding with Tie2, a transmembrane receptor tyrosine kinase expressed mainly on the endothelial cells (Hawighorst et al., 2002; Eklund and Olsen, 2006; Kook et al., 2014). Notably, increasing numbers of studies have found the overexpression of ANG1 and/or ANG2 in a variety of malignant tumors, such as breast cancer, papillary thyroid carcinoma, gastric cancer, and colorectal cancer, while the roles of both proteins in tumor progression and prognosis are controversial (Fagiani and Christofori, 2013; Aktas et al., 2017; Hong et al., 2017; Michael et al., 2017; Ye et al., 2018). Tumor-induced peritumoral lymphangiogenesis has been reported to enhance the lymphatic dissemination of tumor cells to the regional lymph nodes (Beavis et al., 2016). Recent evidence has indicated that ANG1 and ANG2 play a crucial role in the development of the lymphatic system, with the capacity to completely rescue the lymphatic defects in mice lacking ANG2 (Gale et al., 2002). In addition, our previous studies demonstrated the expression of ANG1 and ANG2 in tumor cells of human cervical cancer (Yang, et al., 2017a) and that the higher serum ANG1 / ANG2 ratio was significantly related with longer progression-free survival and overall survival in cervical cancer patients (Yang et al., 2017b). However, the correlation between ANG1 as well as ANG2 expression in cervical cancer and LNM remains unclear. In this study, we analyzed the expression levels of ANG1 and ANG2 in 124 cervical cancer cases and for the first time identified ANG1 as an independent protective factor of LNM in early stage cervical cancer, thereby shedding light on its potential therapeutic role as a predictive biomarker in cervical cancer prognosis.

Material and methods

Patients and sample collection

This study included patients who received radical hysterectomy and systematic pelvic lymphadenectomy due to primary cervical cancer between 2012 and 2014 at Wuhan Union Hospital or the First Affiliated Hospital of Shihezi University. The diagnoses including LNM status were pathologically confirmed, while the exclusion criteria were preoperative chemotherapy, radiation therapy, and a history of other malignant tumors or vascular-associated diseases. The specimens were fixed in 4% paraformaldehyde immediately after resection from patients, and embedded in paraffin within 24 h. The present study was approved by the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology (IORG0003571).

Clinical data

We searched the medical records of these patients in archived files to collect clinical and pathological characteristics, including age at diagnosis, International Federation of Gynecology and Obstetrics (FIGO) stage, pathological type, tumor differentiation, deep stromal invasion (DSI), pelvic LNM, tumor size, and lymph-vascular space invasion (LVSI). In this study, DSI was defined as cervical cancer with a depth of invasion greater than 1/2 full layer.

Immunohistochemistry (IHC)

IHC was performed on 4-μm sections of paraffin-embedded specimens using the PV-9003 Kit (ZSGB-BIO, Beijing, China) according to the manufacturer's

Table 1. Characteristics of included patients.

Clinicopathological parameters	Number (%)
Age (years)	
Median (range)	47.5 (28-76)
<45	49 (39.5)
≥45	75 (60.5)
FIGO stage	
IA1-IB1	65 (52.4)
IB2-IIB	59 (47.6)
Tumor size	
<2cm	13 (10.5)
≥2.0 cm and <4.0 cm	50 (40.3)
≥4cm	34 (27.4)
Unknown	27 (21.8)
Histological type	
Squamous cell carcinoma	108 (87.1)
Adenocarcinoma	16 (12.9)
Differentiation	
Grade 1-2	80 (64.5)
Grade 3	23 (18.5)
Unknown	21 (16.9)
Deep Stromal Invasion (DSI)	
Negative	44 (35.4)
Positive	39 (31.5)
Unknown	41 (33.1)
Lymph-Vascular Space Invasion (LVSI)	
Negative	97 (78.2)
Positive	27 (21.8)
Lymph node metastasis (LNM)	
Negative	95 (76.6)
Positive	29 (23.4)
ANG1 expression	
Negative	49 (39.5)
Positive	75 (60.5)
ANG2 expression	
Negative	35 (28.2)
Positive	89 (71.8)

FIGO, International Federation of Gynecology and Obstetrics.

instructions. In brief, the sections were baked at 65°C for 2 h, deparaffinized in xylene, rehydrated through a gradient of ethanol (100-95-75%), and washed with phosphate buffered saline (PBS). The sections were microwaved in citrate buffer (pH 6.0) for 15 min for antigen retrieval and then cooled down to room temperature naturally. After blocking endogenous peroxidase with 3% H_2O_2 for 10 min at room temperature, tissue sections were incubated with primary polyclonal antibodies against ANG1 (5 μg/mL, AF923, R&D) and ANG2 (5 μg/mL, AF623, R&D) overnight at 4°C respectively. Secondary antibody incubation was carried out with the horseradish peroxidase polymer for 25 min at room temperature, and the positive expressions of ANG1 and ANG2 were detected by diaminobezidin (DAB, ZSGB-BIO) staining. The images were collected using a microscope in three randomly selected fields under 200× magnification. The positive status of ANG1 and ANG2 was defined as brown-yellow particles in the cytoplasm of tumor cells.

Statistical analysis

All data analyses were performed using SPSS Statistics version 23.0 (IBM Corporation, Armonk, NY, USA). Chi-square or Fisher's exact test was used to analyze the correlations between ANG1 as well as ANG2 expression level and multiple clinicopathological characteristics. Univariate and multivariate logistic regression analyses were conducted to develop a predictive model for LNM. The associations between LNM and the variables, including age at diagnosis, FIGO stage, tumor size, histological type, differentiation, DSI, LVSI, and expression of ANG1 and ANG2 were assessed by univariate analysis. Subsequently, factors significantly correlated with LNM

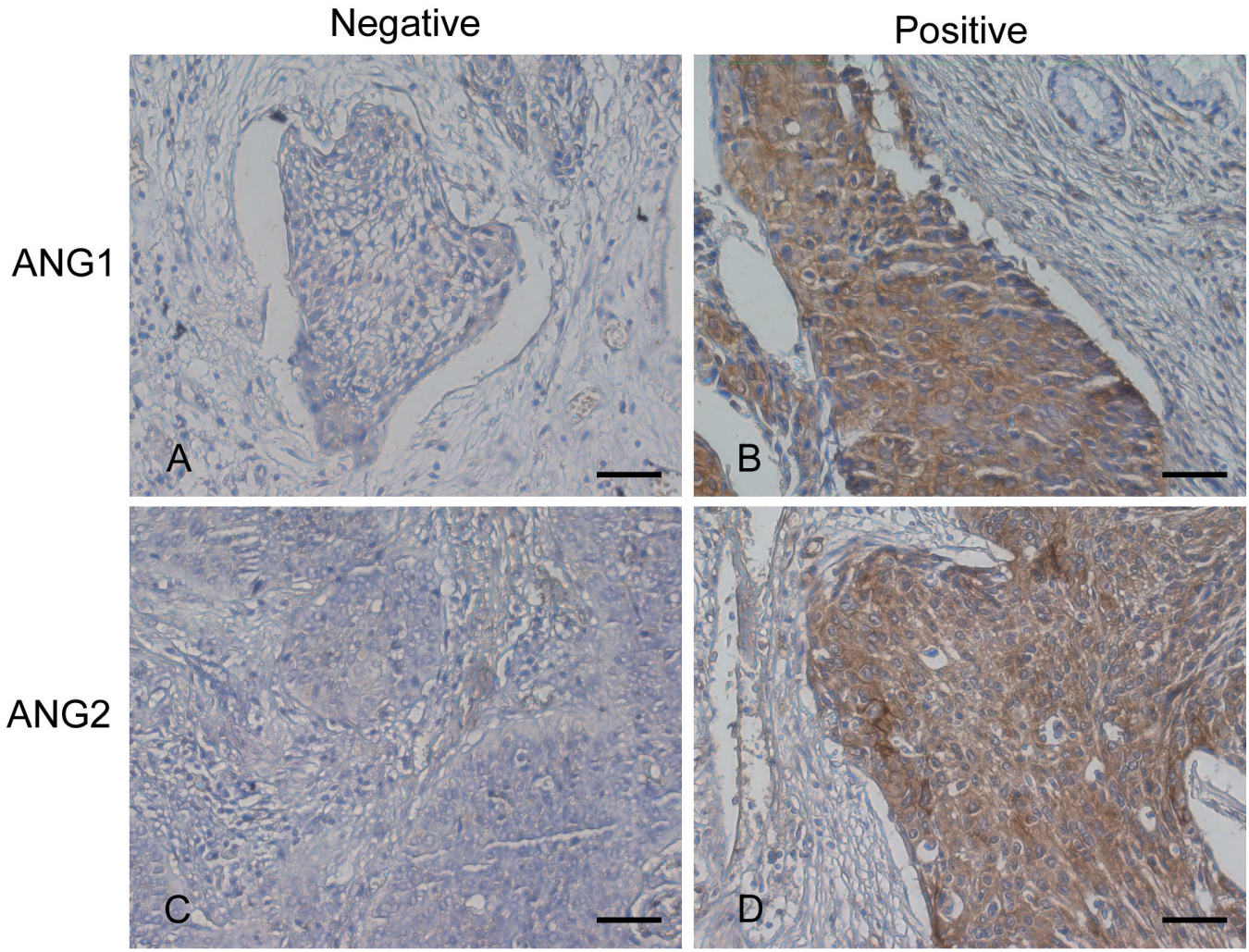

Fig. 1. Immunohistochemistry staining of ANG1 and ANG2 in cervical cancer tissues; representative images are shown. **A.** Negative expression of ANG1 in cervical cancer cells. **B.** Positive expression of ANG1 in cervical cancer cells. **C.** Negative expression of ANG2 in cervical cancer cells. **D.** Positive expression of ANG2 in cervical cancer cells. Both proteins are primarily expressed in the cytoplasm of tumor cells in cervical cancer. Scale bar: 50 μm.

were inputted in a multivariate analysis for identification of the variables associated with independent prognosis values for LNM. All P values were based on two-tailed statistical analysis, and P<0.05 was considered statistically significant.

Results

Expression of ANG1 and ANG2 in cervical cancers

In total, 124 patients with stage IA-IIB cervical cancer were included and their clinicopathological characteristics are summarized in Table 1. IHC staining showed that both ANG1 and ANG2 were predominantly expressed in the cytoplasm of tumor cells (Fig. 1). Positive expression of ANG1 and ANG2 was observed in 75 (60.5%) cases and 89 (71.8%) cases respectively.

As shown in Table 2, ANG1 expression had a significantly negative relationship with LNM (P=0.008), but no correlation was observed in terms of age at diagnosis, FIGO stage, tumor size, histological type, pathological grade, DSI, and LVSI. In addition, we could not find any association between ANG2 expression and the clinicopathological parameters mentioned above.

Table 2. Correlations between ANG1 and ANG2 expression and various clinicopathological characteristics of cervical cancer.

Characteristics	ANG1 expression		P	ANG2 expression		P
	Negative (%)	Positive (%)		Negative (%)	Positive (%)	
Age (years)						
<45	19 (38.8)	30 (61.2)	1.000	10 (51.0)	39 (49.0)	0.154
≥45	30 (40.0)	45 (60.0)		25 (50.7)	50 (49.3)	
FIGO Stage						
IA1-IB1	23 (35.4)	42 (64.6)	0.361	21 (32.3)	44 (67.7)	0.323
IB2-IIB	26 (44.1)	33 (55.9)		14 (23.7)	45 (76.3)	
Tumor Size						
<2cm	3 (23.1)	10 (76.9)	0.283	3 (23.1)	10 (76.9)	0.579
≥2.0 cm and <4.0 cm	24 (48.0)	26 (52.0)		17 (34.0)	33 (66.0)	
≥4cm	16 (47.1)	18 (52.9)		8 (23.5)	26 (76.5)	
Histological Type						
Squamous Carcinoma	40 (37.0)	68 (63.0)	0.174	29 (26.9)	79 (73.1)	0.384
Adenocarcinoma	9 (56.2)	7 (43.8)		6 (37.5)	10 (62.5)	
Differentiation						
Grade 1-2	34 (42.5)	46 (57.5)	0.812	24 (30.0)	56 (70.0)	0.799
Grade 3	11 (47.8)	12 (52.2)		8 (34.8)	15 (65.2)	
Deep stromal invasion						
Negative	17 (38.6)	27 (61.4)	1.000	10 (22.7)	34 (77.3)	0.801
Positive	16 (41.0)	23 (59.0)		10 (25.6)	29 (74.4)	
Lymph-vascular space invasion						
Negative	35 (36.1)	62 (63.9)	0.182	24 (24.7)	73 (75.3)	0.084
Positive	14 (51.9)	13 (48.1)		11 (40.7)	16 (59.3)	
Lymph node metastasis						
Negative	31 (32.6)	64 (67.4)	0.008	26 (27.3)	69 (72.3)	0.814
Positive	18 (62.1)	11 (37.9)		9 (31.0)	20 (69.0)	

Table 3. Univariate and multivariate logistic regression analysis of lymph node metastasis in patients with cervical cancer.

Parameters	Univariate analysis			Multivariate analysis		
	OR	95% CI	P	OR	95% CI	P
Age (years), ≥45 vs. <45	0.625	0.270～1.447	0.272	-	-	-
FIGO stage, IB2-IIB vs. IA1-IB1	2.612	1.097～6.219	0.030	2.298	0.539～9.795	0.261
Tumor size, >4cm vs. ≤4cm	2.400	0.918～6.273	0.074	-	-	-
Histological type, adenocarcinoma vs. squamous cell carcinoma	1.107	0.328～3.736	0.870	-	-	-
Differentiation, Grade 3 vs. 1-2	1.712	0.629～4.658	0.292	-	-	-
Deep Stromal Invasion, positive vs. negative	3.958	1.350～11.608	0.012	1.878	0.428～8.245	0.404
Lymph-vascular space invasion, positive vs. negative	20.662	7.203～59.276	<0.001	34.313	5.914～199.092	<0.001
ANG1 expression, positive vs. negative	0.296	0.125～0.702	0.006	0.107	0.020～0.567	0.009
ANG2 expression, positive vs. negative	0.837	0.338～2.074	0.701	-	-	-

OR, odds ratio; CI, confidence interval; -, not analyze.

ANG1 is an independent protective factor of lymph node metastasis in cervical cancer

In the univariate regression analysis shown in Table 3, we discovered that advanced FIGO stage (odds ratio [OR] 2.612, P=0.030), deep stromal invasion (OR 3.958, P=0.012), and lymph-vascular space invasion (OR 20.662, P<0.001) were positively associated with LNM in patients with early stage cervical cancer, whereas elevated ANG1 expression level in tumor cells (OR 0.296, P=0.006) was negatively related to LNM. Furthermore, multivariate analysis demonstrated that LVSI (OR 34.313, P<0.001) was an independent risk factor of LNM. However, the expression of ANG1 (OR 0.107, P=0.009) was an independent protective factor of LNM.

Discussion

In this study, we demonstrated that the expression of ANG1 in malignant cells, but not ANG2 was an independent protective factor of LNM, which indicates that ANG1 may play a vital role in tumor progression and serve as a novel predictive biomarker for the prognosis of early stage cervical cancer. To the best of our knowledge, this is the first study to evaluate the clinical significance of ANG1 and ANG2 expression in the tumor cells of cervical cancer.

As one of the main pathways of cervical cancer metastasis, LNM is a frequent event at the initial management of patients (Grigsby et al., 2001; Li et al., 2016), and considered as a significant independent risk factor for poor outcome in cervical cancer (Ho et al., 2004; Kidd et al., 2010). Furthermore, the newly updated FIGO staging system has suggested that cases presenting pelvic or para-aortic LNM should be assigned to stage IIIC, regardless of other findings, which highlights the significance of the lymph node status in the stratification and determination of the appropriate treatment approach for patients. Here, we showed that ANG1 expression was negatively correlated with LNM, whereas there was no obvious association between ANG2 expression and the clinicopathological factors of those cases. The relationships between ANG1 or ANG2 protein expression and the clinical features of other types of cancers have been reported in previous studies. Kang et al. revealed that high expression of ANG1 was positively correlated with LNM in papillary thyroid carcinoma (Kang et al., 2017), while the results of Hong et al. showed no correlations between ANG1 expression and the clinical factors in patients with colorectal cancer and that all correlations were negative except for LNM with respect to ANG2 (Hong et al., 2017). This evidence suggests the diverse functions of ANG1 and ANG2 in different types of tumors.

Our results demonstrated that the expression of ANG1 and ANG2 occurred in 60.5% and 71.8% of the patients who were included in the study respectively. This finding further confirmed the existence of ANG1 and ANG2 secreted by cervical cancer cells in larger patient populations compared with our previous study that reported higher levels of ANG1 and ANG2 in cervical cancer epithelial cells than those in the normal cervical epithelia (Yang et al., 2017a). In addition, there have been multiple studies that discovered the elevated level of serum ANG1 or ANG2 in patients suffering from malignancies, indicating their values as circulating biomarkers for diagnosis and prognosis (Gardizi et al., 2012; Aktas et al., 2017; Zhao et al., 2017). Compared to those studies, we elucidated the exact source of ANG1 and ANG2, which are derived from tumor cells, adding evidence for the clinical utility of therapeutic strategies targeting the ANGs-Tie2 pathway. Nevertheless, there are also certain limitations to our research. As a retrospective study, the sample size is relatively small and squamous carcinoma represents the majority of cervical malignancies, accounting for approximately 87.1% of all cases. Expanding the number of the cases diagnosed as adenocarcinoma and other infrequent histological types is needed, which might enhance the statistical power of our results or provide new insight into the roles of ANG1 and ANG2 in cervical cancer.

In summary, our study emphasizes the expression patterns of ANG1 and ANG2 in cervical cancer cells and highlights the possibility that ANG1 may serve as a novel predictor for LNM and a potential therapeutic target for cervical cancer. However, further studies elucidating the regulation of ANG1 in cervical cancer are required.

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Competing interests. All the authors declare that they have no competing interests.

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