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Original Article

CyclinD1 promotes lymph node metastasis by inducing lymphangiogenesis in human ovarian carcinoma

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Abstract: Aims and background: CyclinD1 regulates the G1/S phase transition of the cell cycle and is frequently overexpressed in many types of human cancers. Much evidence has implicated that the expression of CyclinD1 is related to the lymphatic metastasis of human ovarian carcinoma. However, the mechanism of CyclinD1 in lymphatic metastasis of ovarian carcinomas is still unclear. The objective of the present study was to assess the incidence of CyclinD1 expression in ovarian carcinomas and look for its correlation with lymph vessel density (LVD) and clinicopathological variables. Methods: We assessed the expression of CyclinD1 levels and lymph vessel density (LVD) quantified through D2-40 by immunohistochemistry from 110 Chinese patients with primary ovarian carcinomas and 40 with benign ovarian tumors as controls. Results: CyclinD1 was detected in 52 primary ovarian carcinomas (47.3%), which was significantly higher than its expression in the benign ovarian tumors. CyclinD1 expression was correlated with tumor grade, FIGO stage, T stage and lymphatic metastasis. Moreover, the LVD counts in the group of CyclinD1 positive expression were higher than in the group of CyclinD1 negative expression. Conclusions: Our findings indicate that CyclinD1 might be involved in lymph node metastasis by inducing lymphangiogenesis in human ovarian carcinoma.

Keywords: CyclinD1, lymph vessel density, lymphangiogenesis, ovarian carcinoma

Introduction

Ovarian carcinoma is the leading cause of death in all gynecological cancers. Owing to the lack of specific symptoms in the early stages, most cases of human ovarian carcinoma are diagnosed at an advanced stage. Usually, the treatment of ovarian carcinoma is performed by a combination of surgical resections and chemotherapy. The identification of novel therapeutic strategies with the ability to eliminate ovarian carcinoma in the early stages has become a major challenge.

CyclinD1 has been demonstrated to play an important role in the regulation of cell cycle progression and a variety of tumorigenic processes. Meanwhile, CyclinD1 has been revealed to be an important target for anticancer treatment in cases with CCND1 amplification [1]. However, the high expression of CyclinD1 occurs in the early or late stages, and the relationship between CyclinD1 and lymphatic metastasis in ovarian carcinomas is still controversial. Meanwhile, the mechanism of CyclinD1 in the lymphatic metastasis of ovarian carcinomas is still unclear. Therefore, it is necessarily to identify the expression of CyclinD1 in human ovarian carcinomas. Up to now, there has been no research showing the association between CyclinD1 and lymphangiogenesis in ovarian carcinomas.

In our study, we examined the incidence of CyclinD1 protein expression and lymph vessel density quantified through D2-40 immunostaining in 110 primary ovarian carcinoma cases to identify their correlation with clinicopathological variables for elucidating the mechanisms of lymphatic metastasis in human ovarian carcinomas. At the same time, our previous study showed TTF-1 could activate lymph node metastasis by inducing lymphangiogenesis in ovarian carcinoma [2], and we made a further analysis about the relationship between CyclinD1 and TTF-1 in this study as well.

Table 1. CyclinD1 expression in different histologic subtype of ovarian carcinoma

Variables	CyclinD1	
	+	-
Serous	44	42
Mucinous	4	8
Endometrioid	2	2
Clear cell	1	5
Transitional cell	1	1

Materials and methods

Sample collection

Routine formalin-fixed, paraffin-embedded samples of 110 primary ovarian carcinoma cases diagnosed in the period of January 2005 to June 2016 were obtained from the pathology archives of the department of pathology in our hospital, with the required consent and ethical approval for the proposed research project. All the patients did not receive chemotherapy or radiotherapy before their surgeries.

The 110 cases of ovarian carcinoma included 86 serous carcinoma, 12 mucinous carcinoma, 4 endometrioid carcinoma, 6 clear cell carcinoma, and 2 transitional cell carcinoma, with the patients ranging in age from 30 to 81 years (mean, 52.3 years). The histologic grades were grade 1 in 21 patients, grade 2 in 44, and grade 3 in 45; the pathological stages were stage I in 26 patients, stage II in 22, and stage III-IV in 62; the tumor stages were T1 in 25 patients, T2 in 23, and T3-T4 in 62; the status of the lymph nodes were N0 in 65 patients, N1 in 45; the M stage was M1 in 9 patients and M0 in 101. 40 individuals with benign ovarian tumors served as controls. Each diagnosis was made by at least 2 independent pathologists.

Immunohistochemistry

The tissues were fixed in formalin, embedded in paraffin and 4 um thin sections were cut, then deparaffinized in xylene and rehydrated through a graded series of ethanol concentrations. The antigen retrieval was performed by microwaving the tissues, followed by cooling to room temperature. The immunohistochemical staining was done using the Dako REALTM EnvisionTM Detection system (Envision kit; Gene technology, Shanghai, China) according to the manufacturer's protocol. Briefly, endogenous peroxidase activity was blocked with 3% hydrogen peroxide and the sections were incubated in 10% normal goat serum, then incubated overnight with an anti-CyclinD1 mouse monoclonal antibody (1:100 dilution, Zhongshan Company, Beijing, China) or anti-D2-40 mouse monoclonal antibody (1:200 dilution, Zhongshan Company, Beijing, China) at 4°C. Following incubating with a peroxidase-conjugated secondary antibody, the sections were developed with diaminobenzidine. Finally, the nuclei were counterstained with Meyer haematoxylin, dehydrated with graded ethanols and mounted. Negative controls were obtained by omitting the primary antibody. Tumor samples known to be positive for CyclinD1 and D2-40 antibodies were used as positive controls.

Standard of assessment

Immunohistochemical analysis of CyclinD1 was evaluated based on the previous scoring methods used by Saawarn et al. [3]. A staining index (values 0-12) was obtained as a product of staining intensity (1 = mid; 2 = moderate; and 3 = strong staining intensity) and the proportion of immunopositive tumor cells (1-25% = 1, 26-50% = 2, 51-75% = 3, >75% = 4). The staining indexes 9-12 were considered to represent a strong positive reaction, and the staining indexes 1-4 were considered a weak positive reaction.

The lymphatic microvessels were quantified using the procedure described by Weidner [4]. The three most vascularized areas were detected by D2-40 in so-called hot spots under 40× magnification, followed by counting lymphatic microvessels in each of these areas at a magnification of ×400. Single endothelial cells or clusters of endothelial cells were assessed to be individual lymphatic vessels. The mean value of counts at 400× field (0.30 mm^2) was recorded as the LVD in the corresponding section. Finally, the LVD value was converted into the mean value of lymphatic microvessels/mm^2 for statistical analysis. The immunostaining analysis of CyclinD1 and D2-40 was independently performed by 2 observers without any knowledge of the clinical factors.

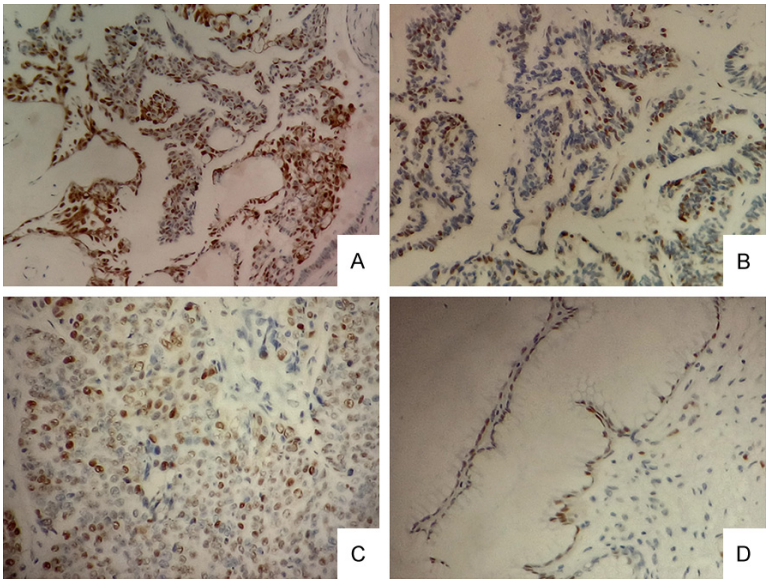

Figure 1. Immunohistochemical staining for CyclinD1: A. Strong positive staining of CyclinD1 in serous carcinoma (×200). B. Faint staining of CyclinD1 in mucinous carcinoma (×200). C. Moderate staining of CyclinD1 in endometrioid carcinoma (×200). D. Faint expression of CyclinD1 in a benign tumor (×200).

Statistical analysis

The correlations between CyclinD1 expression and the clinicopathological characteristics were analyzed by the Fisher's exact test or chi-square test using SPSS version 13.0. *P* values <0.05 were recorded as statistically significant. An unpaired T-test was applied for analyzing LVD counts in different groups and detecting the relationship between LVD and CyclinD1, as well as all clinicopathological parameters. The correlation between CyclinD1 and TTF-1 were performed using Pearson's correlation coefficient.

Results

Expression features of CyclinD1 in ovarian carcinoma and benign controls

In the whole tissue sections (**Table 1**), CyclinD1 nuclear staining was present in 44 of 86 (51.2%) serous carcinomas (**Figure 1A**), 4 of 12 (33.3%) mucinous adenocarcinomas (**Figure 1B**), 2 of 4 (50%) endometrioid carcinomas (**Figure 1C**), 1 of 6 (16.7%) clear cell carcinomas and 1 of 2 (50%) transitional cell carcinomas.

Expression of CyclinD1 in ovarian carcinoma and benign controls

CyclinD1 expression was revealed in 52 of 110 ovarian carcinomas (47.3%) and 11 of 40 (27.5%) benign ovarian tumors. The frequency of CyclinD1-positive cells in the carcinoma tissues was significantly higher than in the benign controls (**Table 2**).

Distribution of CyclinD1 related to clinicopathological parameters in ovarian carcinoma

The correlations between CyclinD1 and the different clinicopathological parameters are identified in **Table 3**. A positive correlation was detected between CyclinD1 expression and lymph node metastasis in ovarian carcinoma. CyclinD1 expression in poor differentiation was higher than in well differentiation and the incidence rate of CyclinD1 in stage III and IV was higher than in stage I and II. The positive rate of CyclinD1 expression was higher in T3 and T4 tumors than in T1 and T2 tumors. Positive CyclinD1 expression was higher in serous ovarian carcinoma (51.2%) than non-serous types of ovarian carcinoma (33.3%). However, there were no significant statistical association between CyclinD1 expression and histologic classification.

Correlation between CyclinD1 and LVD counts

The correlation between the expression of CyclinD1 and LVD counts is shown in **Table 4** (P<0.05). The LVD counts in the group with CyclinD1 positive expression were obviously higher than in group with CyclinD1 negative expression.

Correlation between CyclinD1 and TTF-1

The correlation between the expression of CyclinD1 and TTF-1 is shown in **Table 5** (P>0.05). There was a negative relationship between CyclinD1 and TTF-1.

Discussion

As we expected, a high percentage of CyclinD1 positive expression was identified in human ovarian carcinoma. The positive expression of CyclinD1 in ovarian carcinoma revealed a high-

Table 2. CyclinD1 and LVD count in ovarian carcinoma and benign control

	n	CyclinD1		X^2	P	LVD	F	P
		+	-			Mean ± SD		
Ovarian carcinoma	110	52	58	4.71	0.030	11.47±11.62	3.383	0.001
Benign ovarian tumor	40	11	29			21.63±17.67		

er frequency than in benign ovarian tumors, and a positive association was shown between CyclinD1 and lymph node metastasis as well. Meanwhile, LVD counts were shown to be correlated with the expression of CyclinD1. Therefore, we concluded that CyclinD1 might promote lymph node metastasis in the presence of lymphangiogenesis in human ovarian carcinomas.

The cell cycle is divided into distinct temporal phases, G1, G0, S, G2 and M. CyclinD1 plays an important role in regulating cell cycle progression by complexing with cyclin-dependent kinases (Cdks) to phosphorylate the pRb protein, leading cells from a resting state, G0, through to the G1, S, G2 and M phases. The over-expression of CyclinD1 drives tumor cell proliferation by overriding the G1 checkpoint, resulting in a growth advantage for tumor cells and enhancing tumorigenesis [5]. A large number of studies identified that CyclinD1 was over-expressed in many types of cancer, including lung, head and neck, breast, esophagus, colon and prostate [6]. With respect to ovarian carcinomas, only a limited number of studies on CyclinD1 expression have been published.

The over-expression of CyclinD1 seems to be act as an early event in the development of ovarian carcinomas [7, 8]. Some authors reported that a high level of CyclinD1 correlates with the progression of ovarian carcinomas [9]. The over-expression of cyclinD1 is positively related to pathological grade and may be regarded as a predictive marker for poor prognosis in ovarian serous carcinomas [10]. Recently, a report showed that the expression of the CyclinD1 protein was related to FIGO stage and to histological differentiation in epithelial ovarian cancer tissue [11]. However, there was another study that revealed that CyclinD1 was related to low malignancy of ovarian carcinomas, hinting at a better prognosis [12]. Therefore, the relationship between the expression of CyclinD1 and clinicopathological features in ovarian carcinomas remains unclear. Our study showed that CyclinD1 expression in poorly differentiated tissues was higher than in well differentiated tissues, and the incidence rate of CyclinD1 in stages III and IV was higher than in stages I and II, suggesting that CyclinD1 could serve as a marker for determining poor prognosis in ovarian carcinomas.

As for the relationship of CyclinD1 with lymph node metastasis, it is still controversial. In esophageal squamous cell carcinoma, the percentage of carcinomas with lymph node metastasis is higher in the CyclinD1 positive carcinomas than in the CyclinD1 negative tumors [5]. Other studies showed the expression of CyclinD1 related to lymph node metastasis in epithelial ovarian cancer tissue [11]. However, one study showed that CyclinD1 is unrelated to lymph node metastasis in esophageal squamous cell carcinoma tissues [13]. In our study, the over-expression of CyclinD1 was observed in the lymphatic metastasis group more than in the non-metastasis group, suggesting that CyclinD1 may play an important role in the lymph node metastasis of ovarian carcinomas. Meanwhile, the mechanism of CyclinD1 in the lymphatic metastasis of ovarian carcinomas is still unclear. To our knowledge, no studies have specifically shown an association between CyclinD1 expression and lymphangiogenesis in human ovarian carcinoma. Our study was the first to reveal that a significant relationship was present between the expression of CyclinD1 and LVD in ovarian carcinomas, hinting that CyclinD1 may promote lymph node metastasis of ovarian carcinomas by inducing lymphangiogenesis. Meanwhile, our previous study showed TTF-1 could promote lymph node metastasis in the presence of lymphangiogenesis in ovarian carcinoma [2] and this study showed a negative correlation between CyclinD1 and TTF-1, suggesting that they are independent factors in the lymph node metastasis of ovarian carcinomas.

On the other hand, CyclinD1 has been revealed to be an important target for anticancer treatment in cases with CCND1 amplification [1]. In metastasizing bladder cancer, a high expression of CyclinD1 in lymph node metastases predicted favorable a response to chemotherapy [14]. Both of these studies hinted that it was

Table 3. Distribution of CyclinD1 and LVD related to clinicopathological features

Variables		CyclinD1		X^2	P	LVD	F	P
		+	-			Mean ± SD		
Grade	1	6	15	6.17	0.0458	0.76±11.68	0.174	0.841
	2	19	25			0.99±10.72		
	3	27	18			2.25±11.62		
FIGO Stage	I	3	23	18.27	0.00011	7.24±6.19	3.486	0.034
	II	11	11			0.07±10.10		
	III+IV	38	24			1.47±11.62		
T	T1	4	21	14.83	0.0006	7.54±5.97	3.200	0.045
	T2	10	13			9.38±10.26		
	T3+T4	38	24			3.82±13.26		
N	N0	22	43	11.49	0.0007	8.65±10.06	3.047	0.003
	N1	30	15			5.53±12.61		
M	M0	49	52	0.28	0.599	0.03±10.81	4.753	0.001
	M1	3	6			27.58±7.88		
Histologic subtype	Serous	44	42	2.393	0.122	12.50±12.26	1.780	0.078
	Non-serous	8	16			7.77±8.15		

Table 4. Correlation between CyclinD1 and LVD

		n	LVD	t	P
			Mean ± SD		
CyclinD1	+	52	14.55±13.22	2.664	0.009
	-	58	8.70±9.24		

Table 5. Correlation between CyclinD1 and TTF-1

		TTF-1		X^2	P
		+	-		
CyclinD1	+	17	35	2.723	0.099
	-	11	47		

possible that CyclinD1 could apply in the administration of adjuvant chemotherapy. But there was no related investigation reported on ovarian carcinomas. So, whether CyclinD1 could be applied in targeted treatment of ovarian carcinomas needs further exploring. At the same time, due to the lack of related follow-up data, our study could not elaborate on this. Therefore, larger studies are still needed to identify the function of CyclinD1 expression on the treatment and prognosis of ovarian carcinomas. These will become our next study targets.

In conclusion, the over-expression of CyclinD1 in human ovarian carcinoma is more often detected in patients with poor differentiation, an advanced FIGO stage and lymph node metastasis. There is a significantly positive relationship between the expressions of CyclinD1 and LVD, indicating that CyclinD1 could promote lymph node metastasis by inducing lymphangiogenesis and functioning as a predictor of poor prognosis. Therefore, CyclinD1 will become an important target for inhibiting the lymph node metastasis of ovarian carcinomas in the future.

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Disclosure of conflict of interest

None.

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