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Original Article

Elevated ZEB2 and c-myc expression and their clinical significance in the epithelial ovarian cancer

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Abstract: Previous evidences showed that Zinc finger E-box-binding homeobox 2 (ZEB2) and c-Myc were involved in the pathogenesis of human tumors. Meanwhile, ZEB2 had been reported to induce c-Myc in some carcinomas possibly. The aim of this study is to measure the expression patterns of ZEB2 and c-Myc in ovarian tissues and epithelial ovarian cancer (EOC) and the associations between their combined effects and the pathological features of EOC patients. In this study, we used two tissue arrays, which derived from 208 samples, and evaluated protein expression of c-Myc and ZEB2 by immunohistochemistry separately. Scores ranging from 0 to 9 were obtained by multiplying the percentage of positive cells by intensity (0-3). Immunohistochemical analysis revealed that increased c-Myc and ZEB2 expression displayed in nucleus and cytoplasm of the EOC compared with the ovarian tissues. High expression of c-Myc positively correlated with FIGO stage (P=0.035), histological grading (P=0.039), T stage (P=0.008). High expression of ZEB2 positively correlated with pathological type (P=0.003), FIGO stage (P=0.028), T stage (P=0.002), N stage (P=0.04). Additionally, ZEB2 overexpression positively correlated with c-Myc overexpression in the EOC patients (P<0.001) and the corresponding expression of two oncogenes in EOC patients has a significant correlation with T stage (P<0.001), FIGO stage (P=0.008). Our results suggested that combination of c-Myc and ZEB2 is a potential unfavorable factor for the EOC progression.

Keywords: C-Myc, ZEB, epithelial ovarian cancer, clinicopathological characteristics, protein expression, immunohistochemistry

Introduction

Ovarian cancer is the fifth cause of cancer-related death and the sixth of the most occurrences among women today, which arises from the ovarian surface epithelium [1]. Among the three main types of ovarian cancers, which is epithelial ovarian cancer, sex cord stromal tumors and germ cell tumor, the epithelial tumors account for nearly 90% [2]. Epithelial ovarian cancer (EOC) is considered as the female cancer has high mortality rates after long terms of clinical observation [3]. Whereas unlike the other common gynecology tumors, the factors regulating the proliferation, survival, and metastasis of EOC are still poorly acquired [1]. The patients were mostly detected at their late stages due to the limited early diagnosis criteria [4].

We first focused on ZEB2 (Sip1, Zfhx1b), which belongs to zinc-finger family of transcription factors and plays a crucial role in microRNA-regulated Epithelial-Mesenchymal Transition (EMT) mechanism in diverse carcinomas [5-9]. ZEB2 have been examined its expression in breast cancer cells [10], hepatocellular cancer cells [11], pancreatic cancer [12] et al. *Silvia Prislei et al.* [13] had reported that the nuclear expression of ZEB2 was found to be of relevant prognostic significance in ovarian cancer. Furthermore, a recent study found out ZEB2 may be a biomarker in the ascetic fluid of those patients with advanced grades of serous ovarian carcinoma [14]. However, to our knowledge, the expression pattern of ZEB2 in EOC was still unclear so far.

At the meantime, we focused on another oncogene, c-Myc, which is a member of MYC family of transcription factors and participates in some aspects of cell progression, including metabolism, regulating cell cycle, proliferation, differentiation, apoptosis [15]. The amplifica-

tion of c-Myc had been reported in numerous cancers, such as gastric cancer [16], pancreatic cancer [17], lymphomas [18] et al. Likewise, *Jiabo Di et al.* [19] reported that c-Myc are expressed as a stem cell marker in the ascites cells and tumor tissue of ovarian cancer patients. Further, our prior study has concluded that downregulation of ZEB2 was companied by the decreased expression of c-Myc in glioma [20]. Some early evidences had demonstrated that ZEB2 and c-Myc play a role on the tumorigenesis of cancer, including cancer cell stemness [21, 22]. However, the pattern of their coexpression in EOC remains unrevealed until now. In the current study, immunohistochemistry (IHC) for tissue microarrays were applied to examine the distribution and frequency of ZEB2 and c-Myc expression in the ovarian and EOC cases and their correlation with the clinical pathological parameters.

Materials and methods

Sample collection

Two EOC tissue arrays (208) ranged from 2001 to 2007 were bought from Alenabio Corporation, collected from a numbers of patients who took part in the general survey. All EOC patients had undergone first surgery and experienced no chemotherapy or radiotherapy before surgery. All specimens had confirmed pathological diagnosis and were staged according to the FIGO 2009. The Patients, whose ages ranged from 18 to 81 years old, grouped by two pathological type, containing 192 tumor patients and 16 noncancerous patients, 139 T1 stage patients and 32 T2 stage patients, 21 T3 stage patients, 173 N0 stage patients and 9 N1 stage patients, 182 M0 stage and 10 M1 stage patients.

Immunohistochemistry

Firstly, two paraffin sections (3 μm) from 208 EOC samples were incubated at an oven at 60°C for 4 h. Put them in 100% xylene to routinely dewaxed and rehydrated in graded ethanol series (100%, 90%, 80%, and 70% ethanol) and washed those in distilled water according to standard protocols as previously described [23]. Then the heat-induced antigen retrieval was applied in 10 mM citrate buffer for 3 min at 100°C. After natural cooling, peroxidase blocking reagent containing 3% hydrogen peroxide and serum was to eliminate the endogenous peroxidase activity and nonspecific antigen, followed by incubation with goat anti-human polyclonal ZEB2 antibody (1:50) (Santa Cruz Biotechnology) and c-Myc (1:100) (Santa Cruz Biotechnology) respectively overnight at 4°C. After using phosphate-buffered saline to wash, the slides were incubated with biotin-labeled rabbit anti-goat antibody for 10 min at room temperature and were where after incubated with streptavidin-conjugated horseradish peroxidase (HRP) (Maixin Inc, China). The peroxidase reaction was developed by using 3, 3 diaminobenzidine chromogen solution in DAB buffer substrate. Sections were visualized with DAB, counterstained with hematoxylin, mounted in neutral gum, and analyzed by using a bright field microscope.

Evaluation of staining

The immunostained slides were separately reviewed and scored in a blinded fashion by two pathologists based on the percentage of immunoreactivity and intensity of staining in tissue cells as previous described [24]. When differences occurred, the respective slides were reinvestigated jointly by both investigators. The proportion of positive tumor cells was evaluated as follows: 0 (no positive tumor cells), 1 (<10% positive tumor cells), 2 (10-50% positive tumor cells) and 3 (>50% positive tumor cells). The scoring criteria for staining intensity were graded as: 0 (no staining), 1 (light yellow staining), 2 (yellow staining) and 3 (brown staining). The staining index was calculated by multiplication of proportion and intensity. Using this assessment method, we assessed the expression of ZEB2 and c-Myc in EOC specimens and noncancerous tissues by determining the staining score (0, 1, 2, 3, 4, 6 or 9). A score ≤4 was recognized as low expression and ≥6 as high-level expression.

Statistical analyses

SPSS 20.0 software was applied to perform all statistical analyses. The x^2 test was used to verify the relationship between the clinicopathologic characteristics and the expression of two oncogenes. Spearman test were done, using the H-scores to examine the pairwise comparisons between two oncogenes. A value

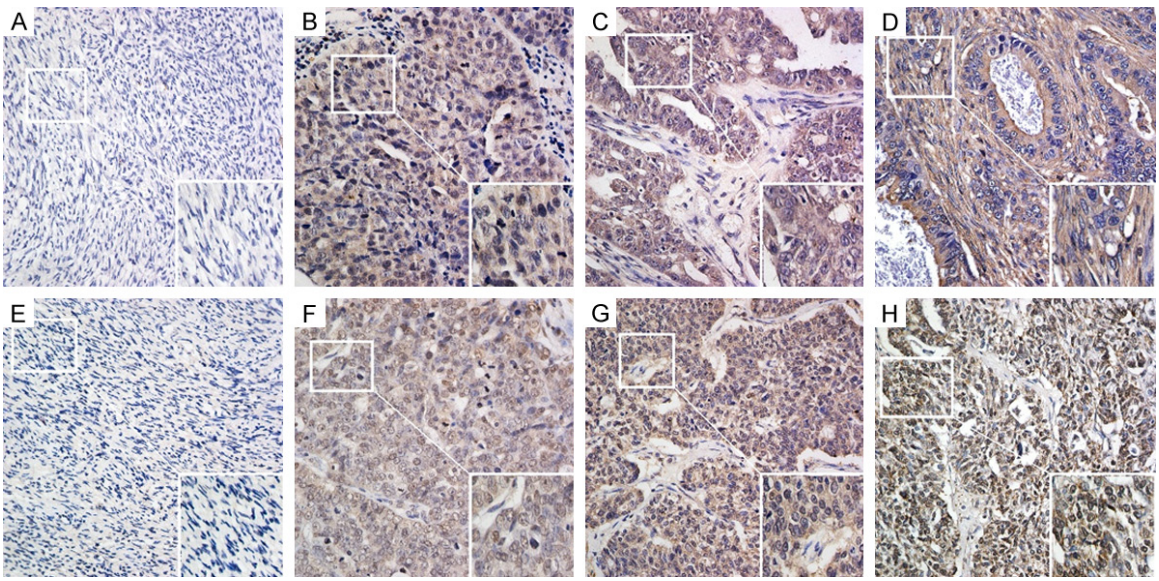

Figure 1. ZEB2 and c-Myc expression in the EOC and ovarian tissues were examined by immunohistochemistry. Negative expression of ZEB2 (A) and c-Myc (E) was demonstrated in a normal ovarian case (400×). Light yellow ZEB2 staining (B) was observed in the nucleus of lower stage EOC case (400×) and light yellow c-Myc (F) staining was observed in cytoplasmic of a well-differentiated EOC case. Yellow ZEB2 (C) staining was observed in cytoplasmic and nucleus of stage III EOC case (400×) and yellow c-Myc (G) staining was observed in cytoplasmic of a stage II EOC case. Brown ZEB2 (D) staining was observed in nuclei of a stage IV EOC case (400×) and brown c-Myc (H) staining was observed in cytoplasmic of a stage IV EOC case.

of less than 0.05 was considered statistically significant.

Results

Expression of ZEB2 in the ovarian and EOC patients

We measured the expression levels and localization of ZEB2 protein in 191 archived paraffin-embedded EOC samples and 13 normal ovarian tissues using immunohistochemical staining (**Figure 1**). ZEB2 expression was observed mostly in the cytoplasm of normal and tumor tissues, staining of tumor cell nuclei was only observed in 7 of all cases. Additionally, ZEB2 protein highly expressed in 49.2% (94/191) of EOC samples, compared with only 30.8% (4/13) of normal samples, which is significantly lower than that in the EOC samples (P=0.007) (**Table 2**).

Expression of c-Myc in the ovarian and EOC patients

We measured the expression levels and subcellular localization of c-Myc protein in 191 archived paraffin-embedded EOC samples and 13 ovarian tissues using immunohistochemical staining (**Figure 1**). The immunohistochemical stain of c-Myc was predominantly localized to the nucleus and cytoplasm of noncancerous and neoplastic tissues and the intense stain in nucleus was observed compared to the weak stain in cytoplasm. Moreover, we observed that in 45.8% (103/191) of EOC samples, c-Myc protein was highly expressed. In comparison, only 15.4% (2/13) of non-cancerous ovarian samples had highly expressed c-Myc protein, significantly lower than that in the EOC samples (P=0.001) (**Table 2**).

Association between the clinicopathological characteristics and ZEB2 expression in EOC patients

As shown in **Table 1**, we observed that overexpressed ZEB2 positively correlated with tumor clinical stage (I-II vs. III-IV) (P=0.028), pathological types, T stage (T1 vs. T2 vs. T3) (P=0.002) and lymph node metastasis (N classification) (P=0.040). Furthermore, we did not find out the significant difference between ZEB2 expression and the patients' age, the histological grading and distant metastases in 191 EOC cases.

Table 1. Correlations between two protein expression and clinicopathological parameters

Variables	ZEB2 (%)				c-Myc (%)			
	N	Low	High	P*	N	Low	High	P*
Age								
<47	138	71 (51.4)	61 (48.6)	1.000	138	67 (48.6)	71 (51.4)	0.268
≥47	53	27 (50.9)	26 (49.1)		53	21 (29.6)	32 (60.4)	
Pathological type								
Serous	159	74 (84.9)	85 (15.1)	0.003	159	72 (45.3)	87 (54.7)	0.417
Mucinous	32	24 (75)	8 (25)		32	17 (53.1)	15 (46.9)	
Histological grading								
G1/1-2	67	36 (53.7)	31 (46.3)	0.549	67	38 (58.2)	29 (41.8)	0.039
G2/G3	124	61 (49.2)	63 (50.8)		124	51 (41.1)	73 (58.9)	
FIGO stage								
I-II	165	89 (53.9)	76 (46.1)	0.028	165	81 (49)	84 (51.0)	0.035
III-IV	26	8 (30.8)	18 (69.2)		26	7 (26.9)	19 (73.1)	
T stage								
T1	139	81 (58.3)	58 (41.7)	0.002	139	73 (52.5)	66 (47.5)	0.008
T2	32	12 (37.5)	20 (62.5)		32	11 (34.3)	21 (65.6)	
T3	20	4 (20)	16 (80)		20	4 (20)	16 (80)	
N stage								
N0	173	92 (53.2)	81 (46.8)	0.040	173	83 (47.8)	90 (52.2)	0.102
N1	18	5 (27.8)	13 (72.2)		18	5 (27.8)	13 (72.2)	
M stage								
M0	182	94 (51.6)	88 (48.4)	0.673	182	96 (52.7)	86 (47.3)	0.740
M1	9	4 (44.4)	5 (55.6)		9	2 (22.2)	7 (77.8)	

FIGO, the International Federation of Gynecology and Obstetrics; G1, well differentiated; G2, moderately differentiated; G3 poorly differentiated. *P values by χ^2 test.

Table 2. Two protein expression in the ovarian and EOC tissues

Variables		c-Myc (%)			ZEB2 (%)			
	N	Low	High	P*	N	Low	High	P*
Tumor	191	88 (54.2)	103 (45.8)	0.001	191	97 (50.8)	94 (49.2)	0.007
Normal	13	11 (84.6)	2 (15.4)		13	9 (69.2)	4 (30.8)	

*P values by χ^2 test.

Association between the clinicopathological characteristics and c-Myc expression in EOC patients

The relationship between clinicopathologic characteristics and c-Myc expression levels in individuals with EOC are summarized in **Table 1**. We observed that the expression level of c-Myc positively correlated with FIGO stage (I-II vs. III-IV) (P=0.035), tumor size (T classification) (T1 vs. T2 vs. T3) (P=0.002), histological grading (G1/1-2 vs. G2/3) (P=0.039) in EOC patients. However, we did not find a significant association of c-Myc expression levels with patient's age, the status of pathology classification, lymph node metastasis (N classification), or status of distant metastases (M classification) in 191 EOC cases.

Association between the ZEB2 and C-MYC expression in the EOC patients

As shown in **Table 3**, spearman test evaluated that ZEB2 expression was notably correlated with the c-Myc expression in the EOC patients (P<0.001). As summarized in **Table 4**, a significant P value can been seen in the association between the co-expression level of two proteins on the EOC and tumor size (T stage) (T1 vs. T2 vs. T3) (P<0.001), FIGO stage (I-II vs. III-IV) (P=0.008), pathological types (P=0.036) and lymph node metastasis (N classification)

Table 3. Correlation between the expression of c-Myc and ZEB2 in the EOC

Variables	c-Myc (%)			
	N	Low expression	High expression	P*
ZEB2				
Low expression	99	60 (60.1)	39 (39.9)	<0.001
High expression	92	30 (67.7)	62 (32.3)	

**P* values by Pearson test.

Table 4. The co-expression of two protein in the EOC

Variables	c-Myc & ZEB2 (%)			
	N	LL	HH	P*
Age				
<47	85	43 (50.6)	42 (49.4)	0.899
≥47	37	16 (43.2)	21 (56.8)	
Pathological type				
Serous	99	43 (43.4)	56 (56.6)	0.036
Mucinous	23	16 (69.6)	7 (30.4)	
T stage				
T1	87	52 (59.8)	35 (40.2)	<0.001
T2	21	6 (28.6)	15 (71.4)	
T3	14	1 (7.1)	13 (92.9)	
Histological Grading				
G1/1-2	48	28 (58.3)	20 (41.7)	0.096
G2/3	74	31 (40.3)	43 (59.7)	
FIGO stage				
I-II	105	56 (53.3)	49 (46.7)	0.008
III-IV	17	3 (17.6)	14 (82.4)	
N stage				
N0	110	57 (51.8)	53 (48.2)	0.031
N1	12	2 (16.7)	10 (83.3)	
M stage				
M0	117	94 (49.6)	88 (50.4)	0.673
M1	5	1 (20.0)	4 (80.0)	

FIGO, the International Federation of Gynecology and Obstetrics; G1, well differentiated; G2, moderately differentiated; G3 poorly differentiated; LL, low expression of c-Myc and ZEB2; HH, high expression of c-Myc and ZEB2. **P* values by χ^2 test.

(P=0.031), but not patients' age, distant metastasis, the histological grading. Furthermore, overexpression of ZEB2 and c-Myc was observed in one pair of same samples mainly, especially the advanced stage samples (**Figure 2**).

Discussion

In this study, we systematically examined the expression level of ZEB2 and c-Myc in ovarian and EOC tissues and analyzed the correlations between the expression of these markers and clinicopathological parameters.

ZEB2 is a representative transcription factor that mainly exhibits nuclear and cytoplasmic expression in carcinoma tissues [25]. It has been discovered that ZEB2 staining mainly displayed in cytoplasmic of gastric, bladder, hepatocellular, nasopharyngeal, breast, and laryngeal squamous cell tumors [26-30]. Similarly, our study presented that ZEB2 predominantly expressed in the cytoplasmic of the EOC and normal tissues. Furthermore, we found that expression level of ZEB2 in the EOC tissues is higher than the ovarian tissues. Here the significant associations between high ZEB2 expression and FIGO stage, T stage, pathological type, and lymph node metastasis were identified. These outcomes revealed that ZEB2 can be an unflavored factor in the invasion and metastasis of the epithelial ovarian cancers. The previous study reported that epithelial expression of ZEB2 in the nuclei of carcinomas related with advanced stage and lymph node status in pharyngeal squamous cell carcinoma [31]. Another recent report demonstrated that the cytoplasmic expression of ZEB2 was not associated with clinicopathological variables in hepatocellular carcinoma [32]. Maybe the results varied as the tissues derived from different organs or different races.

Up to date, it is the first study to measure the immunohistochemical staining of c-Myc in the EOC and normal ovarian tissues and its association with the clinical features. Our study showed that c-Myc expression exhibited in the cytoplasmic and nucleus of the EOC tissues and normal tissues, but the strong nucleus expression was observed in most of the samples. Likewise, this result was also consisting with the report by *Sawant et al.* [33]. Whereas *Di et al.* [19] found that c-Myc expressed highly in the cytoplasmic in most of the ovarian cancer cells and a little percentage of ovarian cancer cell were observed in the nucleus through Immunofluorescence staining and microscopy. Additionally, our data had revealed that the c-Myc overexpression was positively correlated with the histological grading, FIGO stage, T stage. This indicated that c-Myc regulated not only the cell differentiation but also the invasion of the EOC. *Sawant et al.* demonstrated that c-Myc had a correlation with

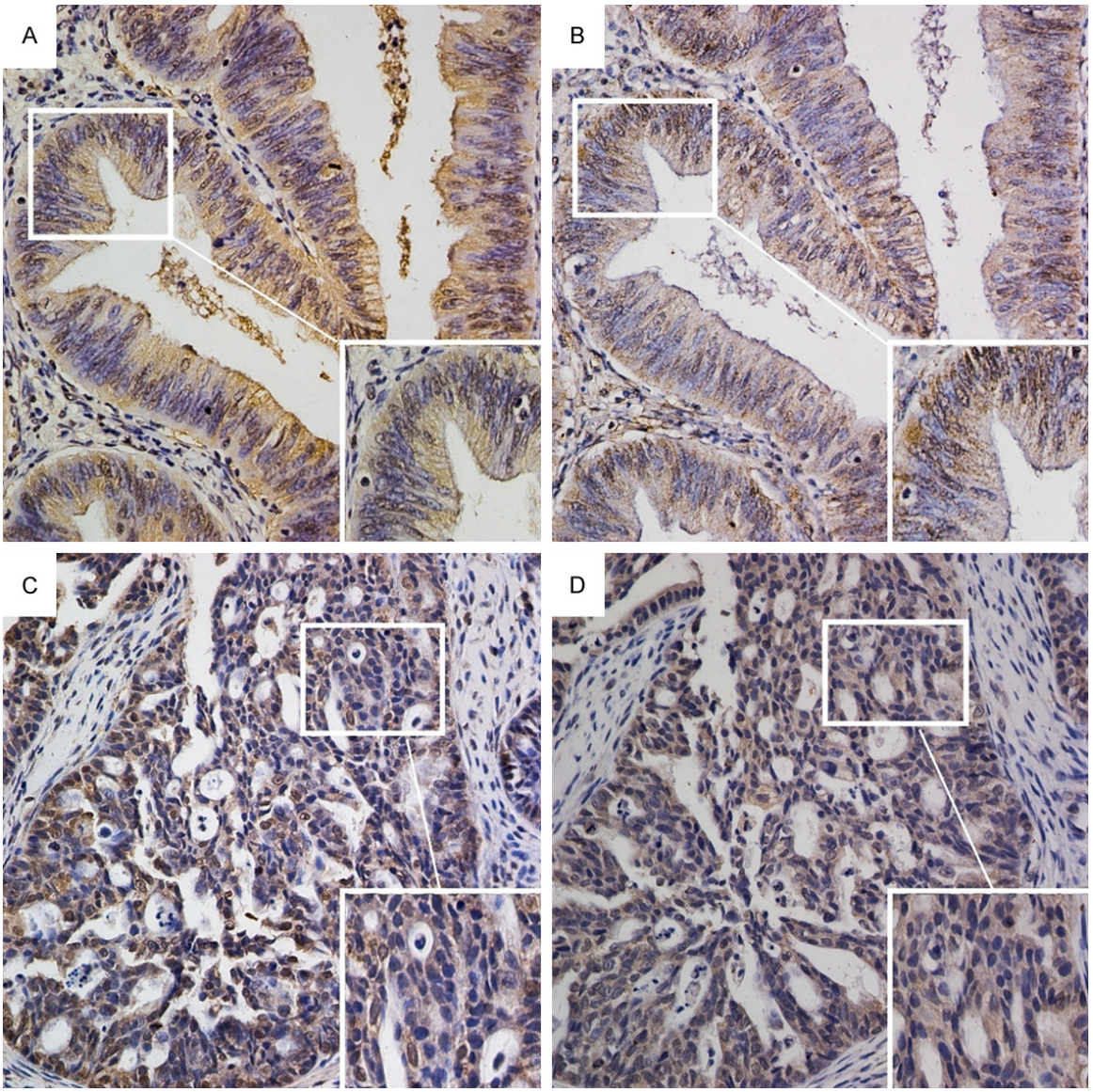

Figure 2. Overexpression of c-Myc (A, C) and ZEB2 (B, D) were observed in the EOC samples. In the same stage II EOC case, the cytoplasmic and nucleus overexpression of c-Myc (A) and cytoplasmic ZEB2 (B) overexpression were observed (400×). In the same stage III EOC case, cytoplasmic and nucleus overexpression of c-Myc (C) and cytoplasmic ZEB2 (D) overexpression were observed (400×).

histopathological grading in the oral cancer. Considering the same race origin of the sample, it may be the different organ tissue or different antibody sources lead to the different results.

To our knowledge, it is the first study to determine the association between the expression of ZEB2 and c-Myc in EOC. Now that spearman test proved that c-Myc and ZEB2 expression is statistically correlated with each other, thus we examined the association between the protein co-expression and clinical pathological parameters. Then we observed that co-expression of both two have significant correlation with T stage, FIGO stage, N stage and pathological types. Furthermore, nearly half (43%) of patients at early stage have high expression of two oncogenes. These findings suggested that c-Myc is important in the diffusion of ZEB2 expression and coexist with it in the progression of ovarian carcinomas.

To focus on the two biomarkers expression on the early stage patients is indeed needed, for the reason that most patients missed the effi-

cient therapy at their early stage for the lack of the practicable criterion. Our data showed that these two biomarkers may be potential indicators for early stage EOC patients. Almost 50% of early stage patients both had high expression of c-Myc and ZEB2. At the same time, here we verified a roughly parallel tendency in the association between the clinical parameters and ZEB2, c-Myc expression. But it is a pity that the present study had not contained the overall survival analysis for the absence of patients' survival information. So we are not able to investigate the prognosis vale of the two biomarkers in the early stage patients.

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Disclosure of conflict of interest

None.

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