EJGO European Journal of Gynaecological Oncology

Role of TWIST2, E-cadherin and Vimentin in epithelial ovarian carcinogenesis and prognosis and their interaction in cancer progression

X. Li[1,2], J. Yang[2], X. Wang[1], X. Li[2], J. Liang[1], H. Xing[1]

[1] Department of Obstetrics and Gynecology, Xiangyang Central Hospital, Hubei University of Arts and Science, Xiangyang, Hubei
[2] Department of Obstetrics and Gynecology, Renmin Hospital, Wuhan University, Wuhan, Hubei (China)

Summary

Globally, most patients are at late-stage when they have been diagnosed with ovarian cancer. Investigating the potential mechanisms involved in tumor progression and prognosis is essential for improving treatment options, outcomes, and survival. *Objective:* This study elucidated the clinico-pathological significance of TWIST2 and the relationship of TWIST2, E-cadherin, and Vimentin expression in the progression and prognosis of epithelial ovarian cancer (EOC). *Materials and Methods:* Immunohistochemical staining was used to quantify the expression and relevance of TWIST2, E-cadherin, and Vimentin in 103 ovarian specimens, including 30 cases of benign ovarian tumors, 30 cases of borderline ovarian tumors, and 43 cases of EOC. *Results:* The expression of TWIST2 in the cytoplasm may help to maintain characteristics of epithelial cancer cells with E-cadherin normal membranous expression, while nuclear TWIST2 induces tumor translation front with membranous expression of Vimentin, which eventually promotes cancer metastasis. Moreover, the upregulation of TWIST2 was also related to the aberrant expression of E-cadherin and the increased expression of Vimentin, which were reported as important indicators of epithelial-mesenchymal transition (EMT). *Discussion:* The data suggested that co-expression of TWIST2/Vimentin was an independent prognostic indicator for both overall survival and disease-free survival by multivariate Cox proportional hazards model. TWIST2 regulates EMT by depriving the epithelial cell phenotype of E-cadherin and endowing the mesenchymal cell phenotype with Vimentin, which may be involved in the progression and prognosis of ovarian cancer, and TWIST2/Vimentin co-expression might be a novel indicator with prognostic potential in EOC patients.

Key words: TWIST2; E-cadherin; Vimentin; Epithelial-mesenchymal transition (EMT); Epithelial ovarian cancer (EOC).

Introduction

Ovarian cancer has one of the highest mortality rates of all gynecologic malignant tumors. More than 70% of patients are late-stage at diagnosis [1], and the five-year survival rate is approximately 19-40% [2, 3]. Locoregional recurrence and distant metastases are ominous events in patients with advanced epithelial ovarian cancer (EOC). Although many efforts have been made to find biomarkers to predict EOC, truly effective clinical biomarkers are rare. Therefore, new and more effective biomarkers are still needed.

TWIST, including TWIST1 and TWIST2, the basic helix-loop-helix (bHLH) transcriptional factor family, has recently been identified as capable of mediating carcinoma metastasis and progression [4-6]. In the last decade most studies have focused on TWIST1 [7-9], which is correlated with epithelial-mesenchymal transition (EMT) in several types of epithelial cancer [5, 7-10]. TWIST2 was previously found to perform the same function as TWIST1 in malignant transformation, invasion and progression, and it has been shown to be significantly upregulated in many different human cancers and cancer cell lines, including melanoma, esophageal squamous cell, breast, lung, colon, kidney, and cervical cancer [11,12]. Gasparotto *et al.* [13] found expression of TWIST2 correlated with the poor prognosis of head and neck squamous cell carcinomas. Zhou *et al.* [14] suggested that TWIST2 is associated with the invasion and metastasis of salivary adenoid cystic carcinoma. Li *et al.* [12] found that TWIST2 is involved in the cervical malignant conversion and tumor metastasis. TWIST2 is also considered an inducer of EMT [11,15].

E-cadherin and Vimentin, which are the epithelial markers and the mesenchymal cell markers, respectively, have reported to be important indicators of EMT [16, 17]. EMT is one of the main mechanisms of tumor metastasis. In the process of EMT, cells acquire molecular alterations that facilitate cell motility and invasion [18]. Mao *et al.* [6] found that the differential cellular distribution of TWIST2 may be associated with breast cancer progression. The cytoplasmic TWIST2 in cancer cells contributes to the maintenance of epithelial cancer characteristics expressing

Revised manuscript accepted for publication September 2, 2014

Table 1. — *Patients' clinical and pathological features.*

Parameter		No.	%
FIGO Stage	Total	103	
	Benign	30	29.1%
	Borderline	30	29.1%
	EOC (I–II)	16	15.5%
	EOC (III–IV)	27	26.2%
Age median 47 (15-83)	Total	103	
	< 50	57	55.3%
	≥ 50	46	44.7%
Pathological grade	Total	43	
	G1	4	9.3%
	G2	18	41.9%
	G3	21	48.8%
Residual tumors	Total	43	
	< 2 cm	<2 cm	65.1%
	≥ 2 cm	15	34.9%

E-cadherin in a non-invasive state, while the nuclear TWIST2 at the cancer invasion front activates EMT to deprive epithelial property of neoplastic cells, thus facilitating invasion and metastasis. However, no published studies have yet examined the role of TWIST2 and the correlation between TWIST2 and EMT in EOC cases.

To understand the role of TWIST2, E-cadherin, and Vimentin in epithelial ovarian carcinogenesis and prognosis and their interaction in cancer progression, in other words, to reveal the novel mechanism most likely contributes to the function of TWIST2 and EMT that are involved in the development of EOC, the author conducted this study and their data suggested that there was a relationship between TWIST2, E-cadherin, and Vimentin, and that the EMT process depends on TWIST2 subcellular location. These results demonstrate an important role of TWIST2 in EOC invasion and indicate that TWIST2 may be a novel indicator for the dissemination and prognosis of EOC.

Materials and Methods

Specimens

The specimens were collected form 103 ovarian tumors patients with cytoreductive surgeries performed at Xiangyang Central Hospital from 2006 to 2010. Patients with EOC had underwent two to eight courses of paclitaxel and nedaplatin chemotherapy after surgery. These specimens included 30 cases of benign ovarian tumors, 30 cases of borderline ovarian tumors, and 43 cases of EOC. Patients who had received radiotherapy or chemotherapy before surgery were excluded from the study. Two pathologists reviewed each specimen in all cases for diagnosis confirmation. Informed consent was obtained from each patient before using the samples. The clinical information is listed in Table 1.

Immunohistochemistry

Immunohistochemical detection of TWIST2, E-cadherin, and vimentin were performed according to the manufacturer's instructions. Representative four-μm serial sections were prepared from 10% formalin-fixed and paraffin-embedded tissue blocks and then rehydrated. Then, 3% H_2O_2 in methanol was used to block endogenous peroxidase activity for 30 minutes at room temperature. Antigen retrieval was performed using the autoclave treatment for two minutes in citrate buffer (pH 6.0). Then, sections were blocked with 10% goat serum at 37°C for one hour. subsequently, they were incubated with anti-TWIST2 (ab57997 applied at 1:200), anti-E-cadherin and anti-vimentin (both applied at 1:200) in a thermostat for 60 minutes at 37°C. Then, the sections were incubated with secondary antibody deal with the universal SP histostain-plus kit for five minutes. Then, the sections were counterstained with hematoxylin. Phosphate-buffered saline was used as the self-negative control and mammary carcinoma specimens were used as the positive control samples.

Evaluation of immunohistochemistry results

Two independent observers who had no prior knowledge of the patient data reviewed the immunohistochemical sections. The most immunoreactive staining results in each sample were selected for quantification. For TWIST2, cytoplasmic, and nuclear immunoreactivity were examined [19]. Cytoplasmic staining was scored from 0 to 3 by its extent and intensity: 0, negative; 1, weak; 2, moderate; and 3, strong. Nuclear staining was performed by counting the percentage of 100 nuclei showing positive immunoreactivity at 400× magnification, and scoring from 0 to 3 as follows: 0, <5%; 1, 5%-20%; 2, 20%-50%; 3, >50%. A score of 2 to 3 was classified as high-level expression and 0 to 1 as low-level expression. Membranous and cytoplasmic staining of E-cadherin and Vimentin were examined for immunoreactivity using the same tissue as TWIST2. E-cadherin *et al.* [20] was primarily located in the cell membrane and partially in the cytoplasm, where brown granules exhibited positive expression, and only cytoplasmic staining was negative, and four categories were defined according to the staining intensity and extent: <10% (-); 10% -50% (+); 50%-90% (+ +); >90% (+ + +). The latter, (+ + +) was regarded as normal expression; any other expression of heterogeneity was regarded as abnormal expression. Immunoreactivity was classified as normal if the specimens exhibited strong or moderate membranous staining with weak or negative cytoplasmic staining. Other expression patterns were regarded as aberrant immunoreactivity. For Vimentin [20], five fields of each slice were randomly selected, and 100 cells were counted per view. In calculating the percentage of positive cells, the membrane and cytoplasm exhibited brown granules in the case of positive expression. The percentages of cells scored by staining were as follows: 0, < 20%; 1, 20%-50%; 2, >50%. According to the staining intensity score: 0, no coloring; 1, light brown to dark brown; 2, the cell coloring percentage score and the staining intensity score were multiplied, and a total score of 0 was recorded as negative (-); a score >0 points was recorded as positive (+).

Follow-up and postoperative treatment

Patients were followed up from the day of cytoreductive surgery to the day of death or to January 1, 2010. Patients in the study did not undergo treatment prior to surgery. Follow-up data for some patients were recorded during the postoperative examination in the present hospital, and the remaining patients were followed up either by telephone or with a letter. The median follow-up time was 25.4 months. Five cases were lost to follow-up. The follow-up rate was 88.4%. All patients were monitored by gynecological examination, routine blood tests, serum cancer antigen (CA125) concentration, and pelvic B-ultrasound every three months in the first year after surgery, every four months in the second year, and every six months thereafter. A chest X-ray, computed tomography scan (CT) or magnetic resonance imaging (MRI) was performed every six months, or immediately when a recurrence or metastasis was suspected. If necessary, a whole-body fludeoxyglucose positron emission tomography scan (PET) was performed. Tumor recurrence was confirmed by at least two imaging findings. Once recurrence was confirmed, further treat-

Table 2. — *Clinico-pathological characteristics of 43 patients with EOC, and its association between TWIST2/E-cadherin/Vimentin overexpression.*

Clinical-Pathological Variables		No.of patients	TWIST2 + (%)	TWIST2 *p* value	E-cadherin + (%)	E-cadherin *p* value	Vimentin + (%)	Vimentin *p* value
Age	< 50	18	12 (66.7)	0.224	7 (38.9)	0.452	8 (44.4)	0.818
	≥ 50	25	12 (48.0)		7 (28.0)		12 (48.0)	
Pathological grade	G1-G2	22	9 (40.9)	0.044	11 (50.0)	0.012	9 (40.9)	0.451
	G3	21	15 (71.4)		3 (14.3)		11 (52.4)	
FIGO Stage	I-II	16	5 (31.3)	0.013	6 (37.5)	0.594	4 (25.0)	0.029
	III-IV	27	19 (70.4)		8 (29.6)		16 (59.3)	
Residual tumors	< 2 cm	28	13 (46.4)	0.090	11 (39.3)	0.198	11 (39.3)	0.194
	≥ 2 cm	15	11 (73.3)		3 (20.0)		9 (60.0)	
Tumor size	< 10 cm	24	14 (58.3)	0.377	8 (33.3)	0.903	11 (45.8)	0.920
	≥ 10 cm	19	10 (52.6)		6 (31.6)		9 (47.4)	
Histological type	Serous	28	18 (64.3)	0.126	9 (32.1)	0.709	13 (46.4)	0.686
	Mucus	2	1 (50.0)		1 (50.0)		0 (0.0)	
	Clear cell	4	1 (25.0)		2 (50.0)		2 (50.0)	
	Endometrial	7	4 (57.1)		1 (14.3)		4 (57.1)	
	Transitional cell	2	0 (0.0)		0 (0.0)		0 (0.0)	

ment, such as a second surgery and palliative chemotherapy or radiotherapy was provided. Overall survival was defined as the interval between the operation and death or the last observation. Disease-free survival was the period from the tumor excision until tumor recurrence or the last examination.

Statistical analysis

All statistical analysis was performed using SPSS 16.0 software. Differences in TWIST2, E-cadherin, and Vimentin expression from the four histological groups of specimens were analyzed using the non-parametric Kruskal-Wallis test and Mann-Whitney test where applicable. The Chi-square statistic was used to analyze the associations between TWIST2, E-cadherin, and Vimentin expression and clinical-pathological characteristics. The Kaplan-Meier method was used for overall survival and disease-free survival, and the log-rank test was used to analyze statistical significances in the groups. Univariate and multivariate analyses were evaluated with the Cox proportional hazards model. Variables with a p-value < 0.05 after the univariate analysis were retained and entered into the multivariate analysis using a forward stepwise selection procedure. For all tests, a p-value < 0.05 was considered to be significant.

Results

Expression of TWIST2, E-cadherin, and Vimentin and their correlation with clinicopathological factors in EOC

The correlation between clinicopathological factors and TWIST2, Vimentin, and E-cadherin expression is shown in Table 2. The overexpression of TWIST2 and abnormal expression of E-cadherin were significantly associated with pathological grade (p = 0.044, p = 0.012, respectively). TWIST2 and Vimentin overexpression were associated with FIGO Stage (p = 0.013, p = 0.029, respectively). However, the expression of E-cadherin was not association with FIGO Stage, and Vimentin was not significantly associated with pathological grade (p > 0.05). The overexpression of TWIST2 and Vimentin and the abnormal expression of E-cadherin were not significantly correlated with patients' age, residual tumor size, primary tumor size and histological type (p > 0.05).

Expression and subcellular localization of TWIST2 in EOC

In order to reveal the role of subcellular localization of TWIST2 in the occurrence and development of ovarian cancer, we divided these patients into three groups, namely the staining of cytoplasm only, the nucleus only, and both the cytoplasm and nucleus, as shown in Table 3. The expression patterns and subcellular localization of TWIST2 in the ovarian cancer cells were mixed nucleus and cytoplasm staining as shown in Figure 1. Table 2 shows that TWIST2 expression was significantly associated with pathological grade and FIGO Stage (p < 0.05). In these ovarian carcinoma specimens, TWIST2 was detected in the cytoplasm only (36/103, 35.0%), the nucleus only (32/103, 31.1%) and both the cytoplasm and nucleus (30/103, 29.1%) (Table 3). Interestingly, the expression of TWIST2 in the cytoplasm only was correlated with FIGO Stage and tumor pathological grade (p = 0.001 and p = 0.021). The expression of TWIST2 in the nucleus only also associated with FIGO Stage (p = 0.001). Statistical analysis showed that both cytoplasm and the nuclear TWIST2 expression level was not significantly associated with either pathological grade or FIGO Stage (p > 0.05). With increased malignancy of the tumor, the expression of TWIST2 in the cytoplasm only and the nuclear only increased (p = 0.001). TWIST2 may be a new candidate for understanding the metastasis and progression of ovarian carcinoma which may also associated with the subcellular localization of TWIST2 expression.

Association of TWIST2, E-cadherin, and Vimentin expression in patients with EOC

To delineate the correlation of TWIST2, E-cadherin, and Vimentin in EOC, the authors performed immunohistochemical staining in 103 cases of samples, including 30 cases of benign ovarian tumors, 30 cases of borderline ovarian tumors, and 43 cases of EOC.

Table 3. — *The expression of TWIST2 in cytoplasm and nucleus of ovarian carcinoma.*

Clinical-pathological variables		Cases	Cytoplasm only		Nucleus only		Both cytoplasm and nucleus	
			+ (%)	*p* value	+ (%)	*p* value	+ (%)	*p* value
Tumor type	Benign	30	4 (13.3)		3 (10.0)		5 (16.7)	
	Borderline	30	9 (30.0)		9 (30.0)		7 (23.3)	
Malignant	I-II	16	5 (31.3)		4 (25.0)		5 (31.3)	
	III-IV	27	18 (66.7)	0.001	16 (59.3)	0.001	13 (48.1)	0.057
Pathological grade	G1-G2	22	6 (27.3)		8 (36.4)		7 (31.8)	
	G3	21	9 (42.9)	0.284	15 (71.4)	0.021	12 (57.1)	0.095

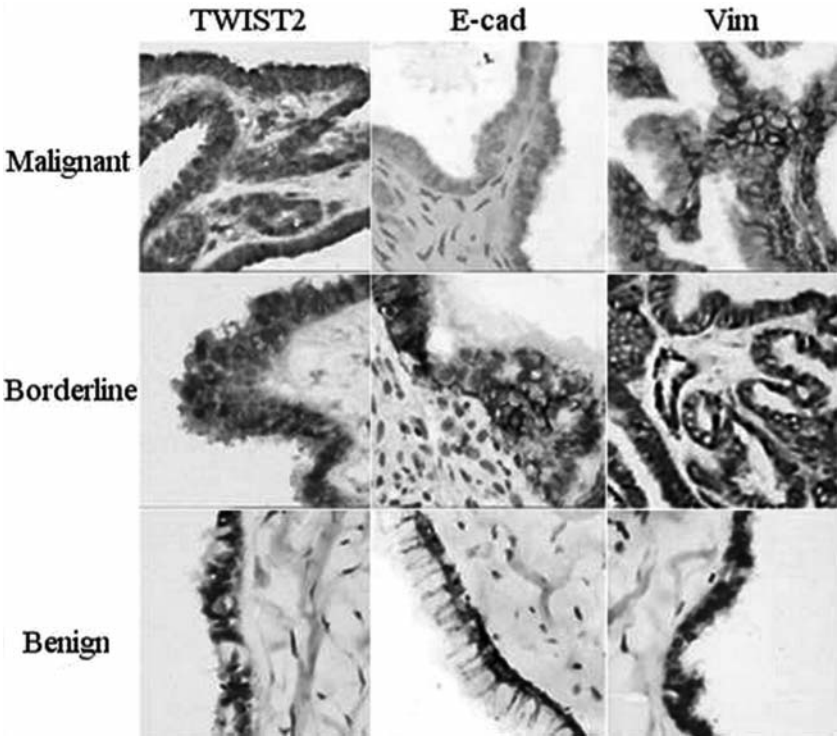

Figure 1. — Immunohistochemistry of TWIST2, E-cadherin and Vimentin in ovarian tissues. With the progression of ovarian carcinogenesis and development of ovarian cancer, the expression levels of TWIST2 and Vimentin were increased and the expression patterns of E-cadherin changed apparently (400× magnification).

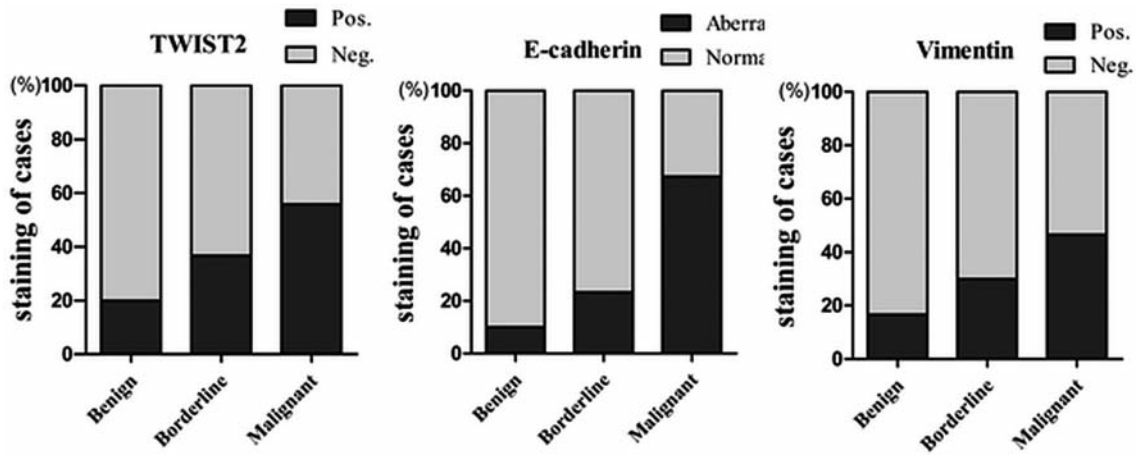

Figure 2. — Summary of TWIST2, E-cadherin, and Vimentin immunohistochemistry expression in ovarian tissues. A, The positive expression rate of TWIST2 gradually increased as the ovarian tumor malignant degree rose. B, Increased aberrant expression of E-cadherin was observed in the progression of ovarian cancer from benign to borderline to malignant. C, As the malignant degree increased, the positive expression rate of Vimentin increased. The *p* values of the Mann–Whitney test for all of the group pairs are exhibited in Table 4.

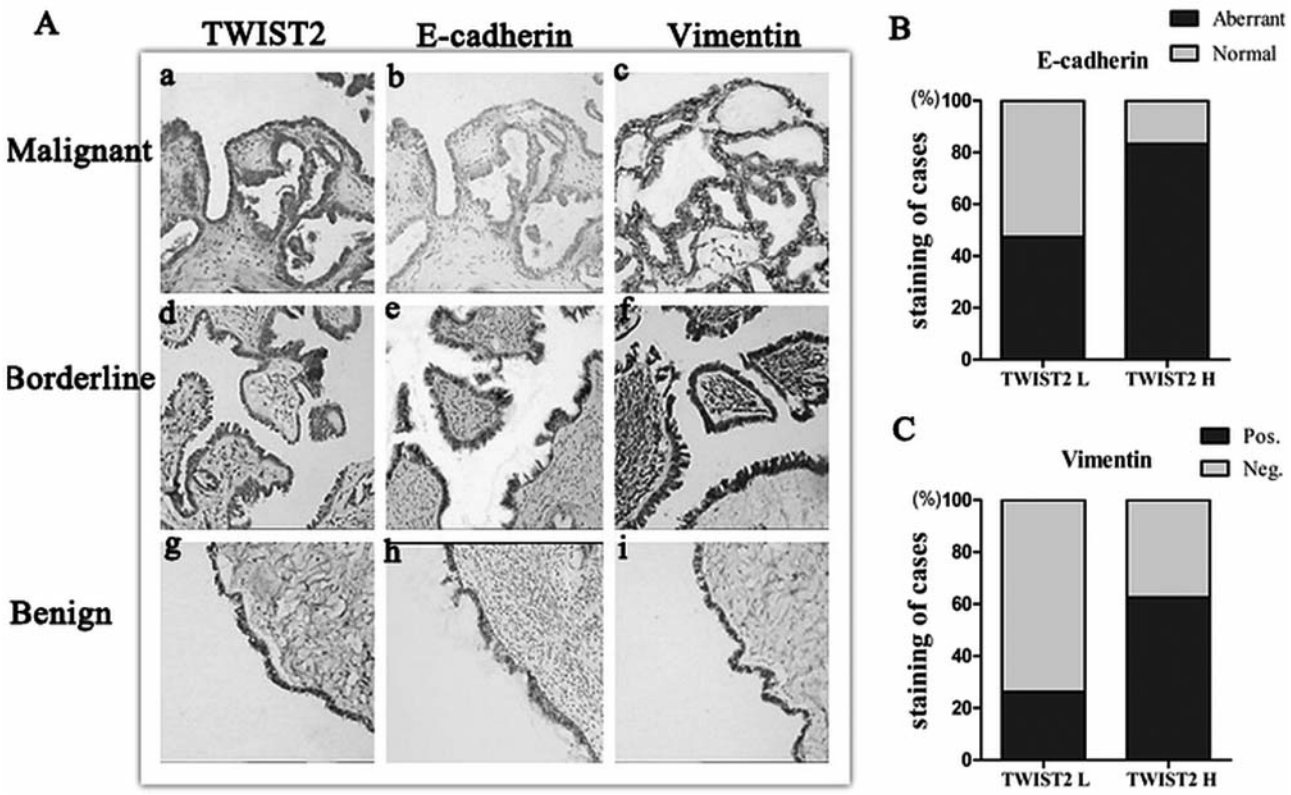

Figure 3. — Association of TWIST2, E-cadherin and Vimentin expression. A, While tissues expressed low levels of TWIST2 and Vimentin, normal expression of E-cadherin was observed in the continuous sections from the same tissues. Further, the high expression of TWIST2 and Vimentin, aberrant E-cadherin staining was shown in the same sections. This association was found during different histological stages: Benign, borderline, and malignant (200× magnification). B, The association between TWIST2 and E-cadherin. The up-regulation of TWIST2 was associated with the down-regulation of E-cadherin, and the decrease of TWIST2 lead to the increase of E-cadherin ($r^2 = -0.276$, $p = 0.003$). C, The association between TWIST2 and Vimentin. Upregulation of TWIST2 was related to the increase of Vimentin, and downregulation of TWIST2 lead to decrease of Vimentin ($r^2 = 0.360$, $p = 0.02$) (r^2 refers to Pearson index). TWIST2 H and TWIST2 L are abbreviations of expression of TWIST2 in high level and low level, respectively. The detailed data are exhibited in Table 5.

Table 4. — *The expression of TWIST2, E-cadherin and Vimentin in EOC.*

Group	Number	TWIST2		E-cadherin		Vimentin	
		(+)	(%)	(+)	(%)	(+)	(%)
Benign	30	6	20.0%	27	90.0%	5	16.7%
Borderline	30	11	36.7%	23	76.7%	9	30.0%
Malignant	43	24	55.8%	14	32.6%	20	46.5%

Table 5. — *Association between TWIST2, E-cadherin, and Vimentin overexpression in patients with EOC.*

	E-cadherin		Vimentin	
TWIST2	-	+	-	+
-	9	10	14	5
+	20	4	9	15

TWIST2 and E-cad: $r^2 = -0.276$, $p = 0.003$; TWIST2 and Vim: $r^2 = 0.360$, $p = 0.02$.

The reactivity of TWIST2 was mainly confirmed by the presence of brown-stained cytoplasm and/or the nuclei of tumor cells, and its expression rates in ovarian benign, borderline, and malignant tumors were (6/30) 20.0%, (11/30) 36.7%, and (24/43) 55.8%, respectively (Figure 2A). The positive expression rate gradually increased as the ovarian tumor malignant degree rose, which was significantly different in the three groups ($p < 0.05$). E-cadherin was primarily located in the cell membrane and slightly in the cytoplasm, and its expression rate for ovarian benign, borderline, and malignant ovarian tumors were (27/30) 90%, (23/30) 76.7%, (14/43) 32.6%, respectively (Figure 2B). The malignant degree increased as the positive expression rate decreased, which was significantly different in the three groups ($p < 0.05$). Vimentin was located in both the membrane and cytoplasm and its expression rates were (5/30) 16.7%, (9/30) 30%, and (20/43) 46.5%, respectively (Figure 2C). As the malignant degree increased, the positive expression rate increased, which was statistically significant different in the three groups ($p < 0.05$), as show in Figures 2 and 3.

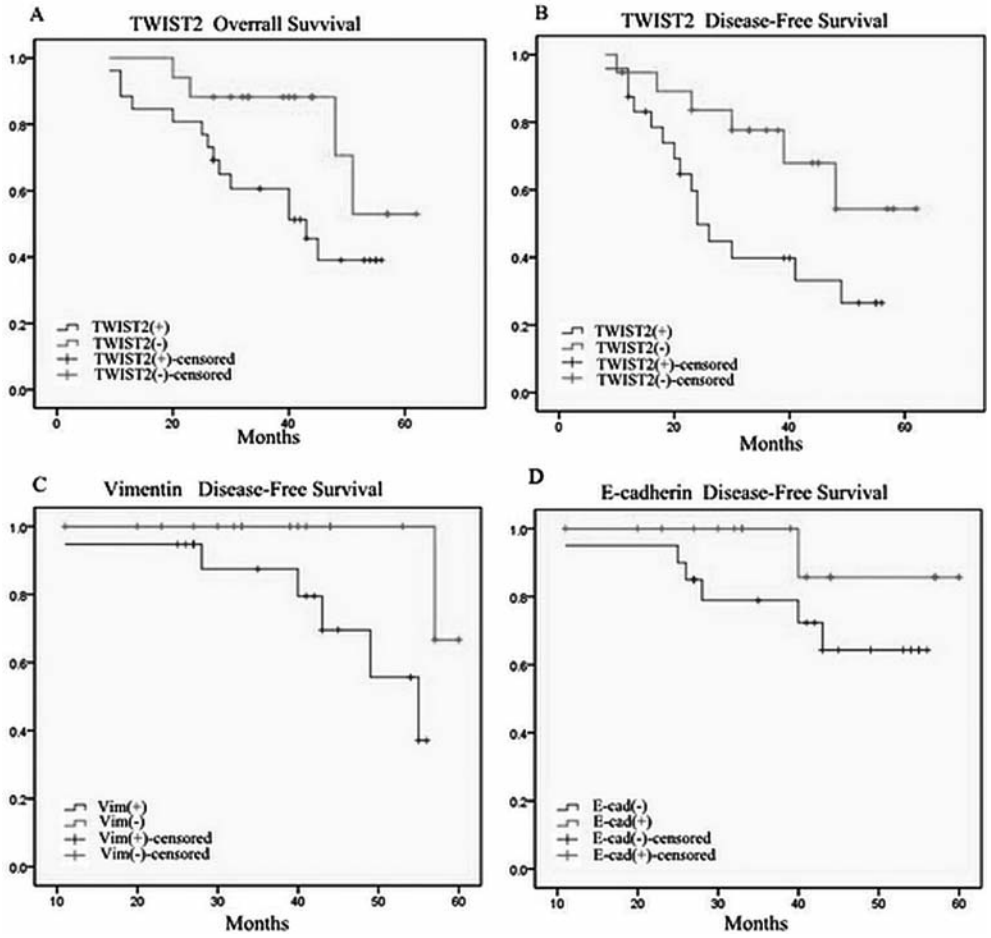

Figure 4. — Kaplan-Meier estimates of overall survival and disease-free survival in EOC patients according to expression of TWIST2, E-cadherin, and Vimentin. A, patients with overexpression of TWIST2 (OS, $p = 0.043$). B, patients with overexpression of TWIST2 (DFS, $p = 0.043$). C, patients with overexpression of Vimentin (DFS, $p = 0.023$). D, patients with aberrent expression of E-cadherin (DFS, $p = 0.155$). Colored dots represent censored subjects. OS: overall survival; DFS: disease-free survival.

The association between TWIST2, E-cadherin, and Vimentin expression is shown in Figure 3. There was a significantly negative correlation between overexpression of TWIST2 and E-cadherin ($r^2 = -0.276$, $p = 0.003$), and there also was positive, but no association of TWIST2 overexpression with Vimentin expression ($r^2 = 0.360$, $p = 0.02$). Figure 3 shows the expression of the three different indicators in the same tissues. The specimens with low expression of TWIST2 displayed significantly high E-cadherin and low Vimentin expression (Figure 3A). When TWIST2 was upregulated, the abnormal E-cadherin and increased Vimentin expression also were enhanced (Figures 3B and 3C). This association was found not only in benign ovarian carcinomas but also in borderline and malignant ovarian carcinomas.

Correlation between overexpression of TWIST2, E-cadherin and Vimentin and prognosis in patients with EOC

To evaluate the prognostic value of TWIST- and EMT-related genes, the authors classified tumors based on relative genes expression levels and used the median as a cutoff value for statistical analysis. The Kaplan-Meier survival analysis revealed a significantly worse overall survival for patients who had overexpression of TWIST2 alone compared with those who had negative TWIST2 staining ($p = 0.043$, Figure 4A). In disease-free survival, overexpression of TWIST2 corresponded to a shorter disease-free survival period ($p = 0.043$, Figure 4B) and the same as Vimentin ($p = 0.023$, Figure 4C). However, E-cadherin had no significant association with either overall survival ($p = 0.195$) or disease-free survival ($p = 0.155$, Figure 4D). Therefore, preliminary results suggest that TWIST2 staining could be a reliable predictor in the prognosis of ovarian cancer patients.

Prognostic factors in patients with EOC

To investigate the accumulative effects of TWIST2, E-cadherin, and Vimentin expression on the prognosis of EOC, the authors divided these 43 patients into three groups according to the positive markers and they were TWIST2/E-cadherin, TWIST2/Vimentin, and E-cadherin/Vimentin. Kaplan-Meier overall survival curves were generated and differences between the three groups were examined. The results showed that patients with overexpression of TWIST2/Vimentin had a worse overall survival

Table 4. — *Univariate and multivariate analyses for the prognostic factors of overall survival and disease-free survival in EOC.*

Prognostic factors		Overall survival				Disease-free survival			
		Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
		HR (95%CI)	*p* value	HR (95%CI)	*p* value	HR (95%CI)	*p* value	HR (95%CI)	*p* value
Age (years)	≥50	0.834 (0.539−1.421)	0.374	NA		0.798 (0.435−1.396)	0.402	NA	
	<50								
Pathological grade	G3	1.887 (1.352−2.468)	0.042	NS		1.631 (1.124−2.366)	0.031	NS	
	G1-G2								
FIGO Stage	III-IV	2.035 (1.394−4.012)	0.034	3.345 (1.384−7.182)	0.002	3.174 (2.142−5.052)	0.021	3.014 (1.213−6.936)	0.004
	I-II								
Residual tumors	≥ 2 cm	2.411 (1.479−4.506)	0.029	NS		2.589 (1.502−5.143)	0.001	2.088 (1.205−4.935)	0.021
	< 2 cm								
Tumor size	≥ 10 cm	0.612 (0.332−1.165)	0.196	NA		0.796 (0.475−1.432)	0.386	NA	
	< 10 cm								
Histological type	Serous	1.468 (1.001−2.583)	0.042	NS		1.538 (1.032−2.761)	0.028	NS	
	Non-serous								
TWIST2	Pos	2.676 (1.173−6.092)	0.015	NS		2.537 (1.157−5.798)	0.018	NS	
	Neg								
Vimentin	Pos	1.616 (1.113−2.326)	0.067	NA		1.537 (1.010−2.337)	0.043	NS	
	Neg								
E-cadherin	Pos	1.090 (0.683−2.215)	0.157	NA		0.982 (0.573−2.012)	0.273	NA	
	Neg								
TWIST2/Vimentin	Co-expression	1.734 (0.952−2.987)	0.031	2.357 (1.573−4.886)	0.026	1.086 (0.799−2.753)	0.028	2.041 (1.897−5.343)	0.033

HR: Hazard ratio; NA: not available; NS: not significant.

($p = 0.023$). A similar result was shown in disease-free survival ($p < 0.001$).

Univariate and multivariate analyses using the Cox's proportional hazards model were performed to predict the prognosis linked factors in all patients. The univariate analysis suggested that patients' pathological grade, FIGO Stage, residual tumors, histological type, and TWIST2/Vimentin co-expression were significantly associated with patient overall and disease-free survival ($p < 0.05$, Table 4). All significant variables ($p < 0.05$) in the univariate analyses were retained for multivariate analyses. Multivariate analysis indicated that FIGO Stage, residual tumors, and TWIST2/Vimentin co-expression were independent and significant prognostic factors in the disease-free survival of all patients ($p = 0.012$, $p = 0.004$, respectively, Table 4). Moreover, TWIST2/Vimentin co-expression was demonstrated to be an independent and remarkable prognostic predictor in the overall survival of all patients ($p = 0.002$). No significant correlation was observed for E-cadherin and Vimentin in overall survival and disease-free survival of patients ($p > 0.05$, Table 4).

The predictive value and application of the TWIST2/Vimentin prognostic model in patients with FIGO Stage and residual tumors in EOC were examined. For FIGO III-IV Stages, patients with overexpression of TWIST2/Vimentin had marginally worse overall survival ($p < 0.001$) and disease-free survival ($p = 0.035$). Patients with residual tumors with overexpression of TWIST2/Vimentin survived for a shorter time in disease-free survival ($p < 0.001$). However, no difference was observed in overall survival ($p = 0.551$). These results suggest that co-expression of TWIST2/Vimentin could be used as a predictor of poor prognosis in patients with FIGO Stage and residual tumors in patients with EOC.

Discussion

In the present study, the results indicated that the overexpression of TWIST2 and Vimentin were significantly associated with FIGO Stage, and the overexpression of TWIST2 and abnormal expression of E-cadherin were sig-

nificantly associated with pathological grade ($p < 0.05$, Table 2). It could be concluded that TWIST2 and Vimentin contribute to the promotion of metastasis. Moreover the expression of TWIST2 may negatively regulate cancer cell differentiation by acting on E-cadherin. The subcellular localization of TWIST2 expression was found in both the cytoplasm and the nucleus (as shown in Figures 1 and 3). Interestingly, the authors found that cytoplasmic expression of TWIST2 was correlated with ovarian cancer cell differentiation ($p = 0.021$). Similar to previous reports [5, 6, 20], preliminary results in this study indicated that cytoplasmic expression of TWIST2 may help to maintain characteristics of epithelial cancer cells with E-cadherin normal membranous expression in a non-invasive state, while nuclear TWIST2 induces EMT in tumor translation front with membranous expression of Vimentin, which eventually promotes cancer metastasis.

In order to further study, immunohistochemistry using a continuous section provided direct evidence of the association of TWIST2, E-cadherin and Vimentin (Figures 1 to 3). The experiment in vitro showed that the upregulation of TWIST2 was associated with the downregulation of E-cadherin and increased Vimentin, and the decrease of TWIST2 led to upregulation of E-cadherin and decrease of Vimentin (Figure 3), which are important EMT indicators [17, 18]. In the process of tumor metastasis, cancer cells obtained an interstitial cell phenotype and acquired invasive ability through EMT, leading to infiltration of the surrounding tissue. In the planting site, cancer cells eventually formed that were similar to the shape and structure of the primary tumors metastases by the transformation of mesenchymal cells and epithelial cells (MET) [21]. Hence, the authors found that TWIST2 may be involved in the ovarian malignant conversion that occurs through EMT by affecting E-cadherin (epithelial marker) and Vimentin (mesenchymal marker) expression.

Crucially, TWIST2 may activate the EMT program, as an inducer of EMT, which is frequently involved in tumor progression [22-27]. However, the way in which TWIST2 participates in EMT of ovarian carcinoma in vivo remains poorly understood. The possible mechanism of TWIST2 involvement in the tumor program was examined and TWIST2 has recently been shown to be an inducer of EMT [11, 28]. In the last decade, EMT has been shown to be a key component of tumor metastasis. However, recent studies on proteins, such as TWIST1, Snail1, and Snail2 that have been involved in EMT suggest that EMT also may contribute to malignant translation other than through invasion of the tumor [15, 29-31]. In addition, TWIST2 has also been extensively involved in the regulation of cellular functions. Recent research indicates that positive expression of HIF-2α was significantly correlated with TWIST2 overexpression in salivary adenoid cystic carcinoma [14]. Furthermore, TWIST2 was also associated with the potential for cancer stem cell self-renewal [16] and methylation [32 ,33], which may reveal patients in the same stage with different prognosis. For EMT, the formation of cancer stem cells are the axis of evil in tumor progression [28], and the present authors conclude that TWIST2 plays a critical role in this axis. Overall, the mechanism of TWIST2's function in EOC is likely to involve multiple links and a complex multi-step process, and TWIST2 mediated mechanism in the development of ovarian cancer implicated with EMT, has yet to be studied in depth.

The present data provided additional knowledge about another function of TWIST2, which correlated with several variables that affected the prognosis of patients. The Kaplan-Meier analysis showed that low TWIST2 expression corresponded to significantly longer overall survival and disease-free survival of patients. Therefore, the results suggest that TWIST2 may play a crucial role in the prognosis of ovarian carcinoma. The present authors also found that patients with co-expression of TWIST2/Vimentin had a worse overall survival and disease-free survival ($p = 0.023$ and $p < 0.001$). Pathological grade, FIGO Stage, residual tumors, histological type, and co-expression of TWIST2/Vimentin were valuable predictors for both overall survival and disease-free survival by univariate analysis. After adjustment by multivariate analysis, FIGO Stage, residual tumors, and TWIST2/Vimentin co-expression were independent and significant prognostic factors in the disease-free survival of all patients, while TWIST2/Vimentin co-expression was demonstrated to be an independent and remarkable prognostic predictor in the overall survival of all patients. The present findings indicate that the assessment of TWIST2/Vimentin co-expression, particularly in FIGO Stage and residual tumors of ovarian cancer, may contribute to the identification of patients at higher risk of fatal progression, making it a potential prognostic marker of EOC.

As far as the present authors know, there is a lack of identification of effective clinical biomarkers and conventional predictors of ovarian cancer. The present study based on protein levels, speculates for the first time that TWIST2 is a significant predictor for determining the metastatic potential and prognosis of ovarian cancer. This discovery may further enrich knowledge about the potential mechanisms involved in tumors and provide additional opportunities for targeted therapy of patients with ovarian cancer.

In summary, the present authors verified that TWIST2 expression was associated with ovarian cancer progression. Importantly, they found that TWIST2/Vimentin co-expression was an indicator with prognostic potential in EOC patients. In this view, TWIST2 may accelerate tumor metastasis by promoting EMT by disrupting E-cadherin and increasing Vimentin expression. The present study should stimulate more exploration of how TWIST2 cooperates in the EMT process, which may be involved in the carcinogenesis, progression, and prognosis of ovarian cancer. Future studies with larger samples and functional experiments are needed to confirm the function of TWIST2 in EOC.

Acknowledgement

This study is supported by Hubei Province's Outstanding Medical Academic Leader Program and Hubei Province Health and Family Planning Scientific Research project (WJ2015MA024&JX6B105).

References

[1] Poveda Velasco A., Casado Herráez A., Cervantes Ruipérez A., Gallardo Rincón D., García García E., González Martín A., *et al.*: "Treatment guidelines in ovarian cancer". *Clin. Transl. Oncol.*, 2007, *9*, 308.

[2] Fotopoulou C., Savvatis K., Steinhagen-Thiessen E., Bahra M., Lichtenegger W., Sehouli J.: "Primary radical surgery in elderly patients with epithelial ovarian cancer analysis of surgical outcome and long-term survival?" *Int. J. Gynecol. Cancer*, 2010, *20*, 34.

[3] Heintz A.P., Odicino F., Maisonneuve P., Quinn M.A., Benedet J.L., Creasman W.T., *et al.*: "Carcinoma of the ovary. FIGO 26th Annual Report on the Results of Treatment in Gynecological Cancer". *Int. J. Gynaecol. Obstet.*, 2006, *95*, 161.

[4] Franco H.L., Casasnovas J., Rodriguez-Medina J.R., Cadilla C.L.: "Redundant or separate entities?—roles of Twist1 and Twist2 as molecular switches during gene transcription". *Nucleic Acids Res.*, 2011, *39*, 1177.

[5] Yuen H.F., Chua C.W., Chan Y.P., Wong Y.C., Wang X., Chan K.W.: "Significance of TWIST and E-cadherin expression in the metastatic progression of prostatic cancer". *Histopathology*, 2007, *50*, 648.

[6] Mao Y., Zhang N., Xu J., Ding Z., Zong R., Liu Z.: "Significance of heterogeneous Twist2 expression in human breast cancers". PLoS One, 2012, 7, e48178.

[7] Zhang Z., Xie D., Li X., Wong Y.C., Xin D., Guan X.Y., et al.: "Significance of TWIST expression and its association with E-cadherin in bladder cancer". *Hum. Pathol.*, 2007, *38*, 598.

[8] Cheng G.Z., Chan J., Wang Q., Zhang W., Sun C.D., Wang L.H.: "Twist transcriptionally up-regulates AKT2 in breast cancer cells leading to increased migration, invasion, and resistance to paclitaxel". *Cancer Res.*, 2007, *67*, 1979.

[9] Niu R.F., Zhang L., Xi G.M., Wei X.Y., Yang Y., Shi Y.R., Hao X.S.: "Up-regulation of Twist induces angiogenesis and correlates with metastasis in hepatocellular carcinoma". *J. Exp. Clin. Cancer Res.*, 2007, *26*, 385.

[10] Rosivatz E., Becker I., Bamba M., Schott C., Diebold J., Mayr D., *et al.*: "Neoexpression of N-cadherin in E-cadherin positive colon cancers". *Int. J. Cancer*, 2004, *111*, 711.

[11] Ansieau S., Bastid J., Doreau A., Morel A.P., Bouchet B.P., Thomas C., *et al.*: "Induction of EMT by twist proteins as a collateral effect of tumor-promoting inactivation of premature senescence". *Cancer Cell.*, 2008, *14*, 79.

[12] Li Y., Wang W., Yang R., Wang T, Su T., Weng D., et al.: "Correlation of TWIST2 up-regulation and epithelial-mesenchymal transition during tumorigenesis and progression of cervical carcinoma". *Gynecol. Oncol.*, 2012, *124*, 112.

[13] Gasparotto D., Polesel J., Marzotto A., Colladel R., Piccinin S., Modena P., et al.: "Overexpression of TWIST2 corre-lates with poor prognosis in head and neck squamous cell carcinomas". *Oncotarget.*, 2011, *2*, 1165.

[14] Zhou C., Liu J., Tang Y., Zhu G., Zheng M., Jiang J., Liang X.: "Co-expression of hypoxia-inducible factor-2α, TWIST2, and SIP1 may correlate with invasion and metastasis of salivary adenoid cystic carcinoma". *J. Oral Pathol. Med.*, 2012, *41*, 424.

[15] Fang X., Cai Y., Liu J., Wang Z., Wu Q., Zhang Z., *et al.*: "Twist2 contributes to breast cancer progression by promoting an epithelial-mesenchymal transition and cancer stem-like cell self-renewal". *Oncogene*, 2011, *30*, 4707.

[16] Radisky D.C.: "Epithelial-mesenchymal transition". *J. Cell. Sci.*, 2005, *118*, 4325.

[17] Thiery J.P.: "Epithelial-mesenchymal transitions in development and pathologies". *Curr. Opin. Cell. Biol.*, 2003, *15*, 740.

[18] Yang J., Mani S.A., Weinberg R.A.: "Exploring a new twist on tumor metastasis". *Cancer Res.*, 2006, *66*, 4549.

[19] Baumgart E., Cohen M.S., Silva Neto B., Jacobs M.A., Wotkowicz C., Rieger-Christ K.M., *et al.*: "Identification and prognostic significance of an epithelial-mesenchymal transition expression profile in human bladder tumors". *Clin. Cancer Res.*, 2007, *13*, 1685.

[20] Yang M.H., Wu M.Z., Chiou S.H., Chen P.M., Chang S.Y., Liu C.J., *et al.*: "Direct regulation of TWIST by HIF-1alpha promotes metastasis". *Nat. Cell. Biol.*, 2008, *10*, 295.

[21] Thiery J.P.: "Epithelial-mesenchymal transitions in tumor progression". *Nat. Rev. Cancer*, 2002, *2*, 442.

[22] Yang J., Mani S.A., Donaher J.L., Ramaswamy S., Itzykson R.A., Come C., Savagner P., *et al.*: "Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis". *Cell*, 2004, *117*, 927.

[23] Yang J., Weinberg R.A.: "Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis". *Dev. Cell.*, 2008, *14*, 818.

[24] Ruan K., Bao S., Ouyang G.: "The mltifaceted role of periostin in tumorigenesis". *Cell. Mol. Life Sci.*, 2009, *66*, 2219.

[25] Thiery J.P., Acloque H., Huang R.Y., Nieto M.A.: "Epithelial mesenchymal transitions in development and disease". *Cell*, 2009, *139*, 871.

[26] Hanahan D., Weinberg R.A.: "Hallmarks of cancer: the next generation". *Cell*, 2011, *144*, 646.

[27] Singh A, Settleman J. EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. Oncogene. 2010; 29(34):4741-51.

[28] Yoshida J., Horiuchi A., Kikuchi N., Hayashi A., Osada R., Ohira S., *et al.*: "Changes in the expression of E-cadherin repressors, Snail, Slug, SIP1, and Twist, in the development and progression of ovarian carcinoma: the important role of Snail in ovarian tumorigenesis and progression". *Med. Mol. Morphol.*, 2009, *42*, 82.

[29] Mikkelsen T.S., Hanna J., Zhang X., Ku M., Wernig M., Schorderet P., *et al.*: "Dissecting direct reprogramming through integrative genomic analysis". *Nature*, 2008, *454*, 49.

[30] Mani S.A., Guo W., Liao M.J., Eaton E.N., Ayyanan A., Zhou A.Y., *et al.*: "The epithelial-mesenchymal transition generates cells with properties of stem cells". *Cell*, 2008, *133*, 704.

[31] Sanchez-Garcia I.: "The crossroads of oncogenesis and metastasis". *N. Engl. J. Med.*, 2009, *360*, 297.

[32] Raval A., Lucas D.M., Matkovic J.J., Bennett K.L., Liyanarachchi S., Young D.C., *et al.*: "TWIST2 demonstrates differential methylation in immunoglobulin variable heavy chain mutated and unmutated chronic lymphocytic leukemia". *J. Clin. Oncol.*, 2005, *23*, 3877.

[33] Mao Y., Toh H.B., Ding Z., Sun L., Hong L., Chen C.S., Wu X.: "Differential methylation of CpG islands within the dermo1 gene promoter in several cancer cell lines". *Oncol. Rep.*, 2011, *25*, 107.

Address reprint requests to:
H. XING, M.D.
Department of Obstetrics and Gynecology
Xiangyang Central Hospital
Hubei University of Arts and Science
No. 39 Jingzhou Street
Xiangyang, Hubei (China)
e-mail: huixingcn@163.com