

Original Article

GLUT-1 and its regulating factor HIF-1 α expression in epithelial ovarian tumors: GLUT-1 is associated with molecular typing and grade of epithelial ovarian cancer

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Abstract: Objective: Hypoxia plays an important role in tumorigenesis and tumor progression. Under the hypoxia environment, glucose uptake is increased to support the growth of tumor. Here we analyzed the expression of GLUT-1 and hypoxia inducible factor-1 α (HIF-1 α) in epithelial ovarian tumors, investigated the correlation between their expression and the molecular typing (Kurman's model) or histological grade of epithelial ovarian cancer. Then explore the possible significance of GLUT-1 in diagnosis and treatment of ovarian cancer. Materials and methods: 154 cases with primary epithelial ovarian tumors (malignant: 63 cases, borderline: 42 cases, benign: 49 cases) were studied. Immunohistochemistry was performed using GLUT-1 and HIF-1 α antibody. GLUT-1 protein expression was quantified by Western blot analysis. GLUT-1 mRNA expression was analyzed by real time PCR (RT-PCR). Results: Both of the expressions of GLUT-1 and HIF-1 α were increased gradually from benign to malignant ovarian tumors, they showed a sequence correlation. But, only GLUT-1 expression synchronously showed a statistical difference in molecular typing, histological grade and FIGO Stage of epithelial ovarian cancer ($P<0.05$). And there is a positive correlation between the expression of GLUT-1 and HIF-1 α , the correlation coefficient was 0.232 ($P<0.05$). At the same time, the expression pattern showed that as the score of GLUT-1 staining increased the intensity and extent of HIF-1 α expression was enhanced accordingly. Western blot and RT-PCR analysis were in accordance with the immunohistochemical staining of GLUT-1. **Conclusion:** Our study suggests that the expression of GLUT-1 was closely related with molecular typing and histological grade of epithelial ovarian cancer. GLUT-1 could be a novel diagnosis biomarker and therapeutic target for epithelial ovarian cancer.

Keywords: GLUT-1, HIF-1 α , epithelial ovarian cancer

Introduction

Epithelial ovarian tumor is the high incidence of gynecology tumor. According to the USA cancer statistics and analysis, in 2016, 1685210 new cancer cases and 595690 cancer deaths are projected to occur in the United States [1]. Therefore, ovarian cancer has become the most fatal gynecologic malignant tumor.

In recent ten years, the understanding of ovarian cancer has made great progress. Based on the distinctive clinicopathologic and molecular genetics feature, a dualistic model of ovarian carcinogenesis has been proposed by Kurman [2], which divided ovarian carcinomas into two groups: type I and type II. This new model reflects the finding that ovarian cancer com-

prises a group of heterogeneous tumors that develop and behave differently. At the same time, a two-tier system about ovarian serous adenocarcinoma has been put forward by Malpica [3], which classified ovarian serous adenocarcinoma into low grade and high grade. These concepts are closely related to assessment of prognosis, individualized treatment and increase of the survival rate.

Compared with the normal tissue, a remarkable feature of malignant tumor is unlimited proliferation. During the over proliferation, a lot of energies consumed which lead to local micro-environment hypoxia and imbalance between energy supply and energy consumption. So hypoxia is one of the basic characteristics of solid tumor microenvironment. In this case, it

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Table 1. Clinicopathologic characteristics of patients

Total Patients		154
Adenoma		49
Serous		21
Mucinous		22
Endometrioid		1
Brenner's		5
Borderline tumor		42
Serous		27
Mucinous		15
Adenocarcinoma		63
Histological type		
Serous	Low grade	9
	High grade	22
Mucinous		12
Endometrioid		9
Clear Cell		11
Histological grade*		
Grade I		19
Grade II		17
Grade III		16
Molecular typing		
Type I		33
Type II		30
Stages		
I		34
II		10
III		19

*Clear cell carcinoma is without histological grading.

needs a large amount of glucose as an energy source. Therefore, with the increase of glucose uptake, metabolism and glycolysis accelerated significantly. This process is mediated by specific transmembranous glucose transporter proteins (GLUTs). GLUT family can be divided into three subfamilies, namely class I (the previously known glucose transporters GLUT1-4), class II (the previously known fructose transporter GLUT5, the GLUT7, GLUT9 and GLUT11), and class III (GLUT6, 8, 10, 12, and the myoinositol transporter HMIT1) [4].

As a housekeeper of GLUTs, GLUT-1 is widely distributed in normal tissues such as erythrocytes and endothelial cells at the blood-brain barrier. Overexpression is considered as self-adaptation in order to achieve rapid growth, proliferation, invasion and metastasis. GLUT-1 can be used as an intrinsic marker to detect

endogenous hypoxia status within the tumor [5]. Although the metabolic consequences of increased glucose transporter are not understood completely, the clinical importance of GLUT-1 expression has been demonstrated. GLUT-1 is primarily undetectable in normal epithelial tissues and benign epithelial tumors. However, GLUT-1 expression has been elevated in a significant proportion of human carcinomas. Various studies have shown a close relationship between GLUT-1 expression and carcinogenesis, tumor development or the unfavorable prognosis of various malignancies [6-8].

Hypoxia inducible factor-1 (HIF-1) is an upstream regulator of GLUT-1, also a key transcription factor to mediate the adaptive response to the hypoxic microenvironment. HIF-1 is an $\alpha\beta$ heterodimeric transcription factor, HIF-1 β subunit is constitutively expressed, under normoxia the HIF-1 α subunit is subject to prolyl hydroxylation, which targets this subunit for ubiquitinylation and subsequent proteasomal degradation [9]. Under hypoxia, however, HIF-1 α degradation is inhibited leading to rapid induction of protein levels and formation of the functional heterodimer [9]. HIF-1 α can regulate over 40 genes expression, for example GLUT-1, P53, VEGF (vascular endothelial growth factor), MDR-1 (multidrug resistance-1) etc. [9], and these target genes are important in tumor energy supply, apoptosis, angiogenesis, drug resistance and recurrence.

In this study we re-evaluate the typical expression pattern of GLUT-1 and its regulator (HIF-1 α) in epithelial ovarian tumors, focused on analyzing the correlation between GLUT-1 expression and Kurman's model and grade. This would contribute to a better understanding of the role of GLUT-1 in ovarian epithelial tumor.

Materials and methods

Patients

A total of 154 patients with primary epithelial ovarian tumor who underwent surgical resection in Tianjin Central Hospital of Gynecology and Obstetrics in 2006 were selected (**Table 1**). None of patients had received preoperative chemo- or radiotherapy. All patients were staged based on the International Federation of Gynecology and Obstetrics (FIGO). Histological type and grade were determined by two of the

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authors, using the World Health Organization (WHO) criteria. The concordance rate was 95% between the 2 pathologists. In case of disagreement, the slides were reviewed simultaneously by the two pathologists seated at a multi headed microscope with a resolution of the difference in opinion. Molecular typing was assigned according to a dualistic model proposed by Kurman [2].

Immunohistochemical analysis

Formalin-fixed, paraffin-embedded tissue blocks were cut into 4 μm -thick sections for hematoxylin and eosin (HE) staining and immunohistochemistry. The expression of GLUT-1 (polyclonal rabbit antibody, diluted 1:200; DAKO, Carpinteria, CA, USA), HIF-1 α (monoclonal mouse antibody, ESEE122, diluted 1:1200; Novus Biologicals, Littleton, CO, USA) were investigated using the standard horseradish peroxidase complex method. The paraffin sections were deparaffinized and immersed in 3% hydrogen peroxidase. Next, an antigen retrieval procedure was performed by microwaving in 10 mM citrate buffer (pH 6.0) at 500 W for 20 min. After washing in phosphate-buffered saline (PBS), the tissue sections were pre-blocked by 10% normal goat serum for 15 min. The protocol for the DakoEnVision™+ kit was followed for each section. The sections were incubated for 60 min with primary antibodies in a humidity chamber. The sections were rinsed with PBS for 15 min, and incubated for 30 min with horseradish peroxidase complex using 3,3'-diaminobenzidine as a chromogen. Counterstaining was performed using hematoxylin. The specificity of the immunohistochemical reactions was checked by omitting the primary antibody. GLUT-1 and HIF-1 α staining results were evaluated by semi-quantity method. GLUT-1 was assessed according to Airley's [10] report, tumor sections were initially scanned at 40 \times magnification so that the distribution of staining could be assessed, then analyzed field-by-field at 200 \times magnification. Membrane-predominant staining was regarded as positive. Each field was assigned a score of 1-4, representative of the approximate area of immunohistochemical staining (0, 0%; 1, 0-5%; 2, 5-15%; 3, 15-30%; 4, >30%). To counteract the effect of variations in staining intensity, only areas of unequivocal staining were include, necrosis, stroma, normal epithelium and distinct edge effects were ignored. The overall scores used in

our study were derived from the average score for all fields. The final scoring scheme was negative (0-1), weak 1+ (1-2), intermediate 2+ (2-3), marked 3+ (3-4). HIF-1 α was assessed according to Wenyan X's method [11], nuclear and/or cytoplasm staining was regarded as positive, the staining intensity and the percentage of stained cells were analyzed. The intensity was scored as 0 (negative), 1+ (weak), 2+ (medium), and 3+ (strong). The percentage of the positive cells was scored as 0 (<5%), 1+ (5%-25%), 2+ (25%-50%), 3+ (50%-75%), and 4+ (>75%). The final score was made by the above score multiplying: negative (0-4), 1+ (5-8), 2+ (9-12).

Western blot analysis

To confirm the immunohistochemical staining result of GLUT-1, Western blot was performed in 20 cases. Using representative cases of adenoma, borderline tumor and adenocarcinoma, the total proteins and membrane protein were extracted and separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to immunobilon-NC membranes (Bio-Rad, Hercules, USA), respectively. GLUT-1 antibody (sc-7903, Santa Cruz Biotechnology, Inc., Texas, USA) was diluted 1:1000 in 5% nonfat milk in PBS-T. Immunoreactivity was visualized by horseradish peroxidase-conjugated anti-rabbit and chemiluminescence. β -actin was used as an internal control. The relative intensity of GLUT-1 is the ratio of GLUT-1 and β -actin gray value which digitalized by GIS1000 analysis software.

RNA extraction and RT-PCR

For cases which were performed Western blot analysis, mRNA expression levels of GLUT-1 were also quantitatively assessed by the RT-PCR. Total RNA was isolated by TRIZOL method, 2 μg RNA was reverse transcribed into cDNA in a 20 μl reaction, and the products were used for real time PCR amplification. The primer of GLUT-1: forward: 5'-GTCTGGCATCAAC-GCTGTC-3', reverse: 5'-CCACAAACAGCGACAC-GAC-3', the length of amplification was 131 bp. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured as a control, the primer of GAPDH: forward: 5'-GTCGGA-GTCAACGGATTG-3', reverse: 5'-CCATGGGTG-GAATCATATTG-3', the length of amplification was 146 bp. The thermal cycling conditions consisted of 95 $^{\circ}\text{C}$ for 10 min, followed by 50

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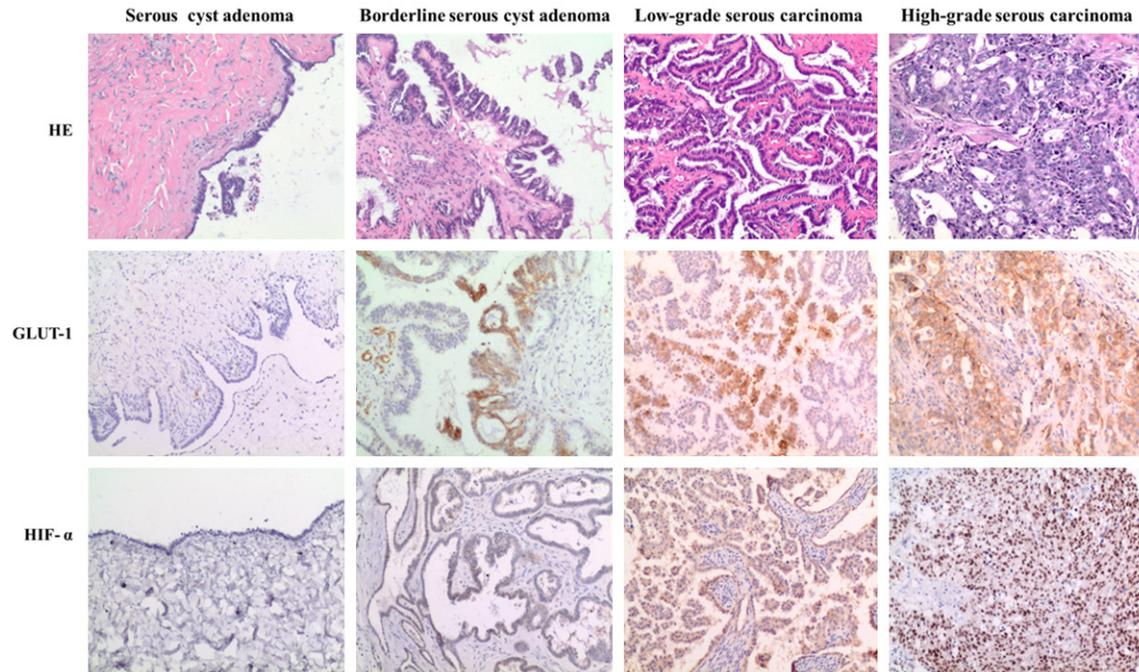


Figure 1. H&E and immunohistochemical staining for GLUT-1 and HIF-1 α in ovarian serous tumors. GLUT-1 positive reaction is seen in both cytoplasm and cell membrane, and HIF-1 α is seen in cytoplasm and nuclei. GLUT-1 and HIF-1 α positive cells are focal or diffuse. Both of them are usually stronger in area of papillary or stratifying structure, as well as, the intensity is increased from adenoma to adenocarcinoma. Original magnifications are all $\times 100$.

cycles of amplification at 95°C for 15 sec and 60°C for 1 min. The relative expression of each mRNA was calculated by the ΔC_t method.

Statistical analysis

Statistical analysis were performed with SPSS 19.0. The Spearman's rank correlation was used to evaluate the relationship between GLUT-1, HIF-1 α expression and neoplastic nature (adenoma, borderline tumor and adenocarcinoma) or clinicopathological characteristics (Molecular typing, grade, histological grade and FIGO stage) in ovarian tumors. The Spearman's rank correlation coefficient was used to evaluate the relationship between GLUT-1 and HIF-1 α expression. $P < 0.05$ were considered statistically significant.

Results

Immunohistochemistry

GLUT-1: The expression was limited to epithelial components, and the stroma was basically negative except for erythrocytes. A positive reaction was seen in both cytoplasm and cell membrane. All of the epithelial ovarian tumors

showed various degrees of positive staining. The pattern of staining cells showed three forms: diffuse, focal or scattered, and usually expressed strongly in the area of papillary or stratifying structure. With the expression increased gradually and the cell membrane became more strongly stained. GLUT-1 staining showed a gradual enhancement with increasing distance from the vascular vessels. Interestingly, we observed giant tumor cells in stroma of ovarian adenocarcinoma which possessed the characteristics of tumor stem-like cells [12] (round, lack branches, at least three times larger than the parental cancer cells) were positive.

HIF-1 α : A positive reaction was most frequently observed in both cytoplasm and nuclei of tumor cells, and the staining pattern were focal or diffuse, similar to GLUT-1, HIF-1 α expressed strongly in the area of papillary or stratifying structure and the area which far from vascular vessels. Positive cells in the stromal were small in number. In general, the staining extent paralleled staining intensity and nuclear labeling. All of the epithelial ovarian tumors showed various degrees of positive staining.

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Table 2. Immunohistochemical analysis of GLUT-1 and HIF-1 α in epithelial ovarian tumors

	GLUT-1				HIF-1 α		
	-	+	++	+++	-	+	++
Adenoma	43	1	4	1	28	16	5
Borderline tumor	29	10	2	1	8	27	7
Adeno-carcinoma	20	14	14	15	8	20	35
<i>P</i> value	1.69 $\times 10^{-8}$ [▲]				2.44 $\times 10^{-10}$ [▲]		

[▲]*P*<0.05.

Table 3. GLUT-1 expression in different clinico-pathologic characteristics

	GLUT-1				<i>P</i> value
	-	+	++	+++	
Molecular typing					
Type I	17	7	7	2	4.92 $\times 10^{-4}$ [▲]
Type II	3	7	7	13	
Grade					
Low Grade	2	3	3	1	0.040 [▲]
High Grade	0	5	5	12	
Histological grade					
Grade I	8	4	6	1	0.015 [▲]
Grade II	3	3	4	7	
Grade III	0	7	3	6	
FIGO Stage					
I	14	6	9	6	0.002 [▲]
II	0	6	3	0	
III	3	4	3	9	

[▲]*P*<0.05.

Here we showed H&E and immunohistochemical staining of GLUT-1 and HIF-1 α in ovarian serous tumors (**Figure 1**). GLUT-1 and HIF-1 α expression are obviously different in benign, borderline and malignant tumor, the intensity is positive related with malignance and histological grade.

In most of the examined cases, HIF-1 α expression was more extensively and uniform than GLUT-1. However, GLUT-1 expression tended to be localized and generally contained in areas where positive for HIF-1 α .

Statistically, a close examination of histomorphological details was focused on analyzing the relationship between GLUT-1 and HIF-1 α expression and neoplastic nature (adenoma, borderline tumor and adenocarcinoma) or clinical-pathological characteristics (Molecular typing, grade, histological grade and FIGO stage) in

Table 4. Correlation between the expression of GLUT-1 and HIF-1 α

GLUT-1	HIF-1 α		
	-	+	++
-	37	33	22
+	4	14	7
++	1	14	5
+++	2	2	13

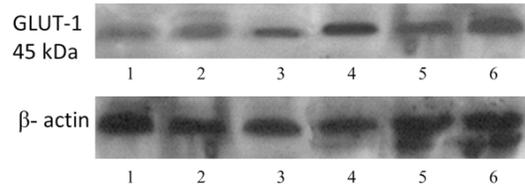


Figure 2. Western blotting analysis of GLUT-1. Lane 1 and 2: adenoma, Lane 3: borderline tumor, Lane 4-6: adenocarcinoma. β -actin is a internal control. Adenocarcinoma showed a broad band around 45 kDa, but the intensity markedly decreased in borderline tumor and particularly in adenoma.

ovarian tumors. With the increase of malignancy of the tumor, the expression of GLUT-1 and HIF-1 α increased gradually (*P*<0.05), they showed a sequence correlation (**Table 2**). Among them, only GLUT-1 expression synchronously showed a statistical difference between Type I and Type II, low grade and high grade serous carcinoma, different histological grade and FIGO Stage (*P*<0.05) (**Table 3**). At the time, there is a correlation between the expression of GLUT-1 and HIF-1 α , the correlation coefficients were 0.323 (*P*<0.05) (**Table 4**). As the score of GLUT-1 staining increased, the extent of HIF-1 α enhanced accordingly.

Western blot

The electrophoretogram indicate that GLUT-1 protein content is different in ovarian tumors, adenocarcinoma showed a broad band around 45 kDa, but the intensity markedly decreased in borderline tumor and particularly in adenoma (**Figure 2**). There is a linear relationship from adenoma \rightarrow borderline tumor \rightarrow adenocarcinoma (**Figure 3**). This result is in accordance with immunohistochemical staining.

Real-time PCR

Quantitative analysis demonstrated that the GLUT-1 mRNA levels are increased gradually from adenoma \rightarrow borderline tumor \rightarrow adenocarcinoma.

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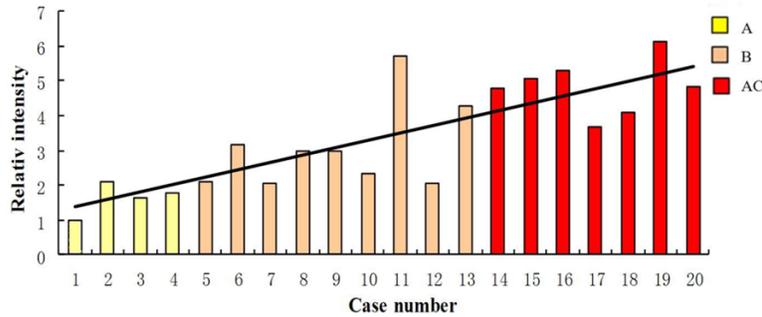


Figure 3. GLUT-1 protein content in ovarian epithelial tumors. GLUT-1 protein content of ovarian tumors is enhanced gradually from adenoma (A) → borderline tumor (B) → adenocarcinoma (AC).

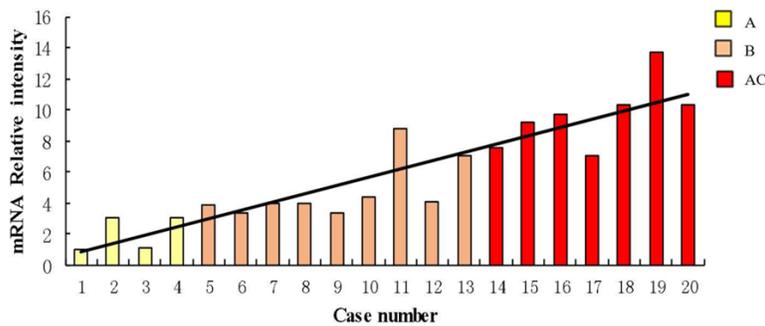


Figure 4. GLUT-1 mRNA expression in ovarian epithelial tumors. GLUT-1 mRNA levels were increased gradually from adenoma (A) → borderline tumor (B) → adenocarcinoma (AC).

rcinoma, and the mRNA levels are positive correlation with protein levels ($r_s=0.454$, $P<0.05$) (Figure 4). Also this result is in accordance with the immunohistochemical expression profiles.

Discussion

In recent years, a lot of studies showed that GLUT-1 expression has been elevated in a significant proportion of human carcinomas. Various studies have shown a close relationship between GLUT-1 expression and carcinogenesis, tumor development or the unfavorable prognosis of various malignancies [6-8]. In this study, we analyzed the expression of GLUT-1 and hypoxia inducible factor-1 α (HIF-1 α) in epithelial ovarian tumors, investigated the correlation between their expression and the molecular typing (Kurman's model) or histological grade of epithelial ovarian cancer. Then explore the possible significance of GLUT-1 in diagnosis and treatment of ovarian cancer.

In our study, the immunohistochemistry results of GLUT-1 and its regulating factor HIF-1 α

showed that i: GLUT-1 expression pattern can be diffuse, focal or scattered in ovarian epithelial tumors; ii: The staining intensity and positive incidence of GLUT-1 in tumor cells is related with blood supply, the farther the stronger (especially in serous tumor); iii: As the staining score increased, cell membrane staining of GLUT-1 tend to be more accentuated; iv: Both GLUT-1 and HIF-1 α are overexpressed at area of papillary or stratifying structure, and gradually enhancement with increasing distance from the vascular stroma, but HIF-1 α expression is more extensively and uniformly than GLUT-1, GLUT-1 expression tend to be localized. All above results are in accordance with Yasuda's [13] study, also from morphology, proved that the tumor cells have the ability to adjust themselves to obtain the energy. As the HIF-1 α expression was uniform staining while GLUT-1 was heterogeneous, especially most of the GLUT-1 positive areas were contained within HIF-1 α positive areas, suggested that GLUT-1 expression is considerably controlled by HIF-1 α expression and GLUT-1 is more sensitive than HIF- α when microenvironment change.

But the expression of GLUT-1 is not regulated by HIF-1 α only, GLUT-1 is influenced by hypoxia, oncogene, antioncogene, glycogen's storage, cell growth factor and hormones etc. The overexpression of GLUT-1 in tumor when microenvironment hypoxia can be induced by two ways, one way is: the reduction of oxygen lead HIF-1 activation, then HIF-1 combined with DNA binding site on GLUT-1 enhancer element to prompt the expression of GLUT-1 mRNA; another way is: hypoxia inhibit mitochondrial oxidative phosphorylation, not only change the intrinsic activity of GLUT-1 but also stimulate the transition of GLUT-1 from storage vacuoles to cell membrane. Mayer [14] discovered that although a robust induction of GLUT-1 by hypoxia has been demonstrated in vitro, this reaction is modulat-

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ed both by confounding factors of the tumor microenvironment and intrinsic traits of malignant cells *in vivo*.

The immunohistochemistry staining result also shown that the expression of GLUT-1 and its regulator are obviously different in ovarian benign, borderline and malignant tumors, the intensity is positive related with the malignance and histological grade. And with the increase of GLUT-1 score, HIF-1 α score increased. These results are accordance with Cantuarria et al [15], Ozcan et al [16] and Ilica et al [17]'s, the overexpression of GLUT-1 and its regulators are related to malignant transformation of ovarian epithelial tumors, then accelerated the metabolism of tumor cell when hypoxia, so GLUT-1 can be utilized as a molecular diagnosis marker of ovarian epithelial tumor in its early stage.

To confirm the accuracy and significance of the immunohistochemical staining profiles, we performed Western blot and real time PCR to analysis the protein and mRNA quantity of GLUT-1. We found that the protein of GLUT-1 is very low in benign tumor, increased in borderline tumor and highest in malignant tumor; the mRNA of GLUT-1 increased with the extent of malignancy, and positive correlation with protein. This result is in accordance with immunohistochemistry staining result. And explain the phenomenon that with the level of malignancy increase, GLUT-1 expression in cell membrane is more obviously. So it is indicate that, under the action of regulator, GLUT-1 mRNA transcription and protein synthesis increase is the early event of malignant transformation of tumors.

Our study also found the expression of GLUT-1 and HIF-1 α differed among the histological types of epithelial ovarian carcinoma, higher in serous carcinoma than mucinous adenocarcinoma, and the difference is statistically significant. This conclusion in accordance with Tsukioke et al [18] and Airley et al [19]'s study. These findings suggest that different histological types of epithelial ovarian carcinoma show different characteristic for glucose uptake, which may be related with the histological structure. In mucinous carcinoma, because glands often show back-to-back arrangement and lack papillary structure, at the same time, the stroma is rich relatively, so the extent of hypoxia is lower. So the positive rate of GLUT-1 shows decreased positive compared with se-

rous carcinoma. This phenomenon can also be seen in thyroid papillary carcinoma and follicular carcinoma [20].

Early this century, based on a review of clinico-pathological and molecular studies, Kurman divided surface epithelial tumors of ovarian into two broad categories designated type I and type II tumors that correspond to two main pathways of tumorigenesis [2]. Our study indicate that GLUT-1 is positive expression in 90% (27/30) of type II carcinoma, and 43.3% (13/30) are strong positive (score 3~4), but the positive rate is 48.5% (16/33) in type I carcinoma, only 2 (2/33) are strong positive, this different is statistical significance. And the expression intensity of GLUT-1 in serous carcinoma is positive correlation with grading. The correlation of GLUT-1 and Kurman typing has been presented for the first time in Ali-Fehmi et al [21] study and their conclusion is consistent with our study. Suggested that type I tumors are slowly growing tumors that might develop from well-established precursor lesion termed borderline tumors, tumor cells are gradually adapt hypoxia microenvironment, with the malignancy increased, imbalance between energy supply and energy consumption increased, GLUT-1 increased either. In contrast, type II tumors grow rapidly and are highly aggressive neoplasm for which well-defined precursor lesions have not been described, microenvironment hypoxia is suddenly and severely, imbalance between energy supply and energy consumption is more serious than type I tumor, at the same time, type II tumors are characterized by mutation of TP53 in 50% to 80% of the cases, reduce the inhibition to GLUT-1 transcription, so GLUT-1 overexpression in order to meet the rapid growth of tumor cells in a short term.

Lots of studies show that hypoxia is a double-edged sword, it is a bad influence factors of tumor therapy, but can provides an important target for tumor of personalized treatment. Because hypoxia is a main feature of tumor cell, hypoxia selective drugs can specific applied in tumor cells, so avoid the resistance to chemotherapy drugs. So we can utilize the characteristic of high expression of GLUT-1 in tumor cell, combine the chemotherapy drugs with GLUT-1 and increase the drug efficiency and specificity. Our study indicate, the expression intensity of GLUT-1 in serous carcinoma is positive correlation with grade, but the positive

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rate of GLUT-1 in low-grade (77.8% (7/9)) and high-grade (100% (22/22)) serous carcinoma showed no statistically significance. Because low-grade serous carcinoma is not sensitive to chemotherapy, so, conjugation of GLUT-1 to chemotherapeutics may be improving the chemotherapy effect of low-grade serous carcinoma, this hypothesis is one of the aspects of GLUT-1 in treatment. If we can suppressing tumor growth by inhibit the transcription of GLUT-1, or kill tumor cells by restrain the activity of GLUT-1 using antisense DNA, RNA may be a new way in therapy of ovarian carcinoma.

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Disclosure of conflict of interest

None.

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