Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading doi:

Expression of serum human epididymal secretory protein E4 at low grade and high grade serous carcinomas

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ARTICLE INFO

Article history:
Received 24 February 2012
Received in revised form 31 March 2012
Accepted 5 Apirl 2012
Available online 20 December 2012

Keywords:
Human epididymal secretory protein E4
Ovarian neoplasms
Heterogeneity
Early diagnosis
Dualistic model
Two-tier grade system

ABSTRACT

Objective: To investigate the value of serum human epididymis protein 4 (HE4) in differential diagnosis of patients with low-grade serous (LGSC) and high-grade serous carcinoma (HGSC) serous ovarian cancer. **Methods:** LGSC and HGSC serous ovarian cancer were diagnosed by the two-tier grade system, serum levels of HE4 and carbohydrate antigen 125 (CA125) were measured by ELISA and radioisotope method, respectively in 60 serous ovarian cancer patients. HE4 and TP53 protein in cancer tissue were measured by immunohistochemical method. **Results:** The difference in density of HE4 and TP53 protein was significant between LGSC and HGSC tissue, while serum CA125 did not show significant difference between different serum samples. There was significant difference in serum HE4 levels between LGSC and HGSC, and the result was different within FIGO (I+II) stage, suggesting HE4 was not a reliable biomarker for the discrimination between LGSC and HGSC. HE4 had potential as a biomarker for the discrimination between LGSC and HGSC but the role in early diagnosis was limited. **Conclusions:** HE4 may be a reliable marker for differential diagnosis of LGSC and HGSC. But its role in early diagnosis of LGSC and HGSC need to be confirmed from the perspective of two-tier grade system.

1. Introduction

The 5-year survival rate of advanced ovarian carcinoma is less than 30%, and on the contrary, the 5-year survival rate of early ovarian carcinoma may be up to 90%. Therefore, early diagnosis is the key to improve prognosis of ovarian carcinomas. As a new tumor marker, the application of human epididymal secretory protein E4 (HE4) in differential diagnosis of ovarian carcinoma has been confirmed[1]. At present, there has been new recognition concerning pathology of ovarian carcinomas, ie. theories of "dualistic pathology model"[2,3] and "heterogeneity of ovarian carcinomas"[4,5] for pathogenic mechanism of ovarian carcinomas. In the past, it has been said that ovarian carcinomas originate from ovary itself, *ie*. ovarian surface epithelium, have the potential for multi-lineage differentiation, and so may form histological types of ovarian tumors, in which serous cystadenocarcinoma is the most common. For the "dualistic pathogenesis", the core is the theory of external origin for ovarian carcinoma, in which it is said that ovarian carcinomas are irrelevant to ovarian surface mesothelium cell, but from plantation outside the ovary. For example, serous tumor is from oviduct mucosa epithelium, endometrioid carcinoma and clear cell tumor are from ectopic endometrium, mucinous tumor and transitional cell carcinoma possibly from para-ovarian Wathard cell nest. Different ovarian carcinomas have different origins. This opinion and the theory of "ovarian carcinomas heterogeneity" supplement each other. According to the latter, epithelial ovarian carcinomas are not

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Foundation project: It is supported by Young Researcher Foundation from Education Department of Jiangxi Province (Grand No. GJJ12161).

a single disease, but a set of diseases of "heterogeneity"[5]. Furthermore, from the molecular view point, the epithelial ovarian cancer may be divided into subtypes Ⅰ and Ⅱ. Low grade serous carcinoma (LGSC) and high-grade serous carcinoma (HGSC) are typical subtypes Ⅰ and Ⅱ ovarian carcinomas, respectively[6]. The two are significantly different in biological behaviors and clinical features[7]: subtype Ⅰ tumor is inert in biological behavior, and the disease will experience a progressive process of ovarian epithelial inclusions-serous cystadenoma-borderline cystadenoma-low grade serous cystadenocarcinoma. Usually, the disease is at an early stage; subtype Ⅱ tumor is characterized as high invasiveness, and is almost always at advanced stage once found. Two subtypes are different in characteristics of molecular genetics: the genetic mutations of KRAS and BRAF etc. occurring in about two thirds of subtype Ⅰ tumor cases, while TP53 mutation was rare; more than 80% of subtype Ⅱ tumor cases were found with TP53 mutation and CCNE1(code: cyclin E1) augmentation. As for the prognosis, among the dead cases of ovarian carcinomas, 25% were of subtype Ⅰ ovarian carcinomas, while 75% were of subtype Ⅱ tumor. Therefore, for serous carcinoma, HGSC and LGSC are two diseases different in biological behavior, pathological mechanism and prognosis though both are serous carcinoma in histology. In recent years, the application of two-tier grading system (also known as MDACC grading system)for ovary serous carcinoma attracted attention[8]. According to traditional three-tier grading system, different grades of ovary serous carcinoma have the same route of tumor occurrence, which cannot reflect the "heterogeneity" of ovarian carcinomas, while two-tier grading system is identical to the dualistic model for occurrence of ovary serous carcinoma, and can guide clinical prognosis[9]. The ultimate goal of differential diagnosis for diseases is to identify diseases different in pathology, type, and prognosis, but identical in clinical manifestation, so as to further guide relevant therapy and prognosis. According to relevant data, diagnosis of ovarian carcinomas based on heterogeneity is more scientific.

Present study also showed high expression of HE4 serous carcinoma and relative low expression of clear cell carcinoma and mucinous ovarian carcinoma[10], which indicated that HE4 was different in diagnosis of different histopathological types. However, at present there is still no literature concerning study on difference of HE4 in low grade LGSC and high grade HGSC of serous carcinoma. According to two-tier grading system, high differentiation serous carcinoma and ovarian endometriocarcinoma are of subtype Ⅱ cancer, clear cell carcinoma and mucinous ovarian carcinomas are of subtype Ⅰ cancer, which indirectly indicate that we should make clear whether there is different expression of HE4 between subtype Ⅰ and subtype Ⅱ cancers, annd whether this difference leads to differential diagnosis of subtype Ⅰ and subtype Ⅱ cancers. In this study, the "two-tier grading system" is used to find out the difference of HE4 in tissue and serum between LGSC and HGSC, as well as the significance in differential diagnosis.

2. Materials and methods

2.1. Subject

Between November 2011 and May 2012, 60 cases pathologically diagnosed as serous ovarian cystadenocarcinoma after operation, who were hospitalized for pelvic mass were selected. According to two-tier grading system for ovary serous cystadenocarcinoma (MDACC grading), they were divided into subtype Ⅰ LGSC and subtype Ⅱ HGSC. And the standard for grading was as Xia *et al*[11]. According to main standard based on morphology of tumor cell nucleus, they were divided into LGSC and HGSC. The low grade nucleus had characters of slight to moderate heteromorphosis, relative identical size, round or oval shape, exquisite chromatin, even distribution, and visible nucleolus; the high grade nucleus was characterized as severe heteromorphosis, different sizes and shapes, coarse chromation, obvious nucleolus, with difference between nucleus sizes$\geqslant$3: 1; for the morphological evaluation of cell nucleus, the region with the most obvious atypia should be selected. Secondary standard based on mitotic count: low grade$\leqslant$12/10 HPF, high grade>12/10 HPF; the counting method was as follows: for region with the most active mitotic figures, counted 10 continuous high power fields (400×), repeated for 4 times, with the one with the most quantity of mitotic figures as the counting result. At the same time, took mutation P53 albumen as the control index. Mostly, subtype Ⅱ was positive in P53 diffusion, while subtype Ⅰ P53 focal positive or negative[9]. The study has been approved by the hospital ethical committee and the patients had informed consent right.

2.2. Methods

The kit for two-step immunohistochemical assay of mouse anti human HE4 monoclonal antibody and mouse anti human TP53 monoclonal antibody and the DAB color development kit were purchased from Beijing Zhongshan Biological Technology Co., LTD. The steps for immunohistochemical staining were performed according to the instruction for use of kit, with the known positive piece as the control (with

HE4 as ovarian carcinomas, and P53 as breast cancer), and PBS solution as substitute of the primary antibody for blank control.

For the serum HE4 enzyme-linked immunosorbent assay (ELISA Double Antibody Sandwich Method), the assay kit was purchased from Fujirebio Diagnostics, Inc of Sweden (Catalog Number 404-10K), unit of concentration: pmol/L, reference value 0-150, instrument: Full automatic enzyme immunoassay analyzer of TECAN Freedom Evolyzerl50. For serum CA125 content, the isotope analyzer and original reagents from the Laboratory Department of our institute were adopted, if serum CA125 value >30.0 IU/mL, the result was positive. Before operation, 2 mL of venous blood was taken from patients, without hemolysis or lipoidemia, and was under 4 000 *g* centrifugation for 5 min. Serum was collected and placed in -70 ℃ refrigerator for storage.

Yellow grain or mass in cytoplasm of tumor cell indicated positive staining of HE4 and TP53 and was measured by a twice- scoring method. Five fields of spot and the region with the densest carcinoma tissues was counted under 400× light microscope, and the average was taken as the intermediate density of this target albumen. Five high power fields was randomly counted, and average positive rate as well as proportion of positive cells in each high power field was calculated. For immunohistochemical semiquantitative analysis, a "13-point" scoring method was used: 0 for colorless, 1 for light yellow, 2 for brown yellow, and 3 for brown. Then it was scored based on proportion of positive cells: 0 for without positive cells, 1 for positive cells <10%, 2 for positive cells 10%-50%, 3 for 50%-80%, and 4 for >80%. Finally, all the scores for staining strength and for positive cells were added for judging the expression results: scores <2 negative, >2 positive, 4-5 moderate positive, 6-7 strong positive.

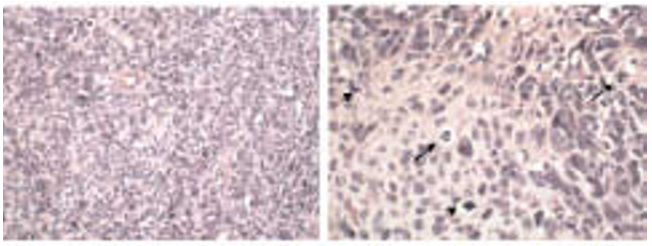
2.3. Statistical analysis

For the categorical data analysis method, chi-square test for paired fourfold data was adopted, and in the test, the samples should be more than 40, and in each grid, the theoretical frequency $T \geqslant 5$. If the samples are more than 40 but the theoretical frequency $T < 5$, a continuous correction formula was used for the chi-square value. If $T < 1$, the exact probability method should be used for calculation of probability. For numeric data analysis, if the data follow the normal distribution, the mean±standard deviation should be used for description, and for comparison between the two, group *t* test should be used. For abnormal distribution, percentile was used for description and rank sum test was used. *Kappa* index reflects consistency between indexes. If *Kappa*>0.75, the consistency is good, and *Kappa* <0.4, the consistency is poor.

3. Results

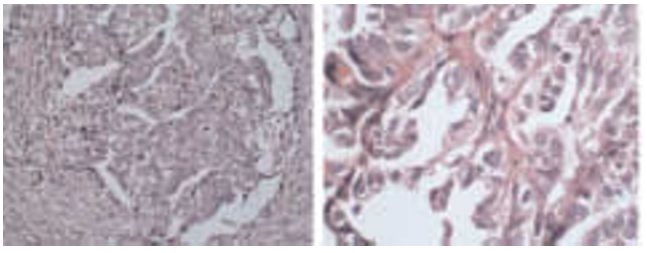
3.1. Grading

LGSC revealed micro papilla structure, with cell nucleus light to moderate heteromorphosis, except for 1 case, mitotic figures <12 /10HPF (average 6/10HPF). For HGSC, borderline lesion transitional region was not observed, 98% of the cases revealed major papilla, fissure like or solid laminate structure, with significant heteromorphosis of cell nucleus, and for all, mitotic figures >12/10HPF(average 37/10HPF) (Figure 1, 2).



1 2

Figure 1. Morphology of Ⅰ/Ⅱ HGSG.
1. HGSG exhibits moderate to marked nuclear atypia. 2. On high power , abundant mitotic activity (arrow), greater than 12 mitoses per 10 high power fields (1.HE×100, 2. HE×400).



1 2

Figure 2. Morphology of Ⅰ LGSG.
1. LGSG was characterized by a micropapillary architecture and clefts surrounding the groups of tumour cells. 2. On high power ,the uniform nuclei with mild atypia and less to 12 mitoses per 10 high power fields (1.HE×200, 2. HE×400).

According to two-tier grading, among 60 cases with ovary serous carcinoma, there were 19 LGSC cases, and 41 HGSC cases. According to WHO grading, among 19 cases with LGSC, 9 cases (47.4%) were of WHO Ⅰ grade, 10 cases (52.6%) of WHO Ⅱ grade; while among 41 cases with HGSC, 24 cases were of (58.5%) WHO Ⅱ grade, 17 cases (41.5%) of WHO Ⅲ grade. Among 19 cases with LGSC, there were 16 at FIGO stage Ⅰ-Ⅱ and 3 at stages Ⅲ or higher; in HGSC patients, there were 5 at FIGO stage Ⅰ-Ⅱ and 36 at stages Ⅲ

or higher. It indicated that there was significant difference between LGSC and HGSC in FIGO staging (χ^2= 29.60, P<0.01).

3.2. TP53 and HE4 immunohistochemical results

In serous carcinoma, the total positive rate of HE4 was 93.33% (56/60), the positive rate of HGSC group was 100% (41/41), and positive rate was 78.94% in LGSG group (15/19). There was statistical significance for the difference (χ^2= 39.05, P <0.01). *Kappa*= 0.837 indicated good consistency of HE4 albumen expression and Ⅰ/Ⅱ classification.

The strong positive rate of P53 was respectively 10.3%(2/19) in LGSG group and 92.7%(38/41) in HGSG group with significant difference (Pearson χ^2= 39.43, P<0.01). *Kappa*=0.810>0.75 indicated good consistency of P53 albumen expression and Ⅰ/Ⅱ classification. For subtype Ⅱ serous carcinoma tumor cell nucleus, the P53 albumen expression was diffusely positive, while for subtype Ⅰ cancer tissues, the focal of TP53 albumen was positive or negative (Figure 3-5).

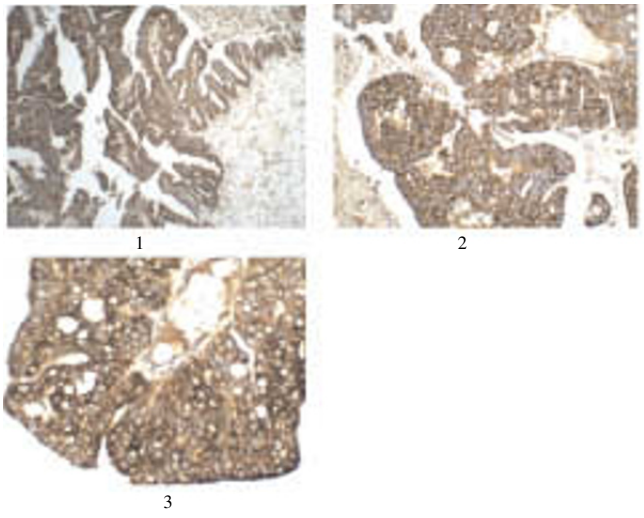


Figure 3. Diffusive positive expression of HE4 protein in type Ⅱ serous ovarian tissue.
1, 2: SP×200; 3: SP×400.

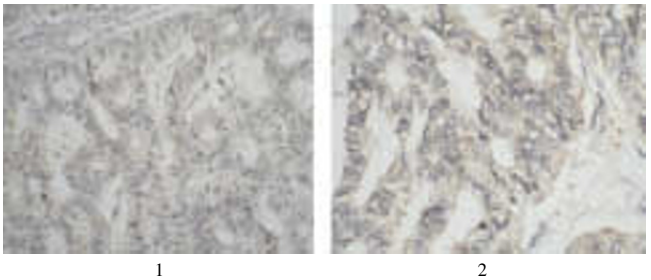


Figure 4. Week positive expression of HE4 protein in type Ⅰ serous ovarian tissue.
1: SP×200; 2: SP×400.

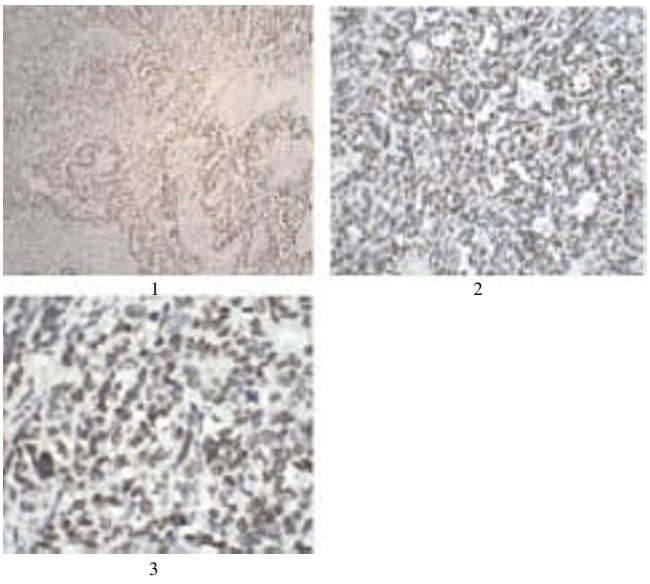


Figure 5. Diffusive positive expression of TP53 protein in HGSG serous ovarian tissue.
1: SP×100; 2:×200; SP3: SP×400.

3.3. Difference between LGSC and HGSC in serum HE4 and CA125

Homogeneity test of variances of samples showed homogeneity of variances in HE4 (F=3.033, P=0.087) and CA125 (F=0.786, P=0.379). Normal distribution QQ test also revealed normal distribution. Independent t test showed the serum HE4 level was (408.23±78.84) pmol/L in HGSC group and, and (288.11±59.52) pmol/L in LGSC group (t=-5.898, P<0.01); serum CA125 level in HGSC group was (475.74±202.34.84) U/L, and in LGSC group (637.0224±324.98 U/L) (t= - 1.987, P=0.052).

4. Discussion

Presents, all literatures indicated the value of HE4 in differential diagnosis and early diagnosis of ovarian carcinoma, but the literatures followed WHO histological classification and FIGO staging, and failed to further analyzed the serous cystadenocarcinoma by subtypes of HGSC and LGSC. It has been proved that HGSC and LGSC are two diseases different in biological behavior, pathological mechanism and prognosis though both are serous carcinoma in histology[6]. Therefore, present literatures failed to be based on "dualistic pathogenesis model", and failed to reflect the "heterogeneity". Therefore, it is necessary to study the expression difference of HE4 HGSC and LGSC.

This study found that there was expression difference of HE4 in tissues and serum between LGSG and HGSG, which indicated that HE4 might conduce to differential diagnosis of LGSG and HGSG. For serum CA125, there was no expression difference between LGSG and HGSG, which indicated that CA125 was not helpful in differential diagnosis of LGSG and

HGSG. This resulted in a thought concerning selection of control index in present diagnostic test: CA125 diagnosis is lack of sensitivity and specificity for ovarian carcinomas, which has been recognized. This study further found that there was no difference for CA125 expression in LGSG and HGSG, which indicated that CA125 might have no value in subtype diagnosis of ovarian carcinomas. This is identical to the results reported. Mao et al reported that CA125 had no effect on differentiation between high grade and low grade serous epithelial ovarian carcinomas[12]. It was proved that 9 serum tumor markers like CA125 showed no difference in expression of different subtypes of (subtype Ⅰ, subtype Ⅱ) ovarian carcinomas[13], which indicated that for evaluating the value of HE4 in differential diagnosis, CA125 was not an ideal control index. There was difference in P53 genetic mutation for subtype Ⅰ and Ⅱ ovarian carcinomas, and some relevant studies showed good correlation of serum p53 antibody (p53AAbs)with "two - tier grading system", which indicated that p53AAbs might be more appropriate as diagnosis control of subtype Ⅱ " ovarian carcinomas". The inert biological behavior of subtype Ⅰ ovarian carcinomas showed relative inert and progressive, and limited to ovary. So when the disease was found, it was usually still at early stage by clinical staging, and it was easy to be found through intravagianal ultrasound and pelvioscopy[3], which indicated that intravagianal ultrasound may be more appropriate as diagnosis control of subtype Ⅰ "ovarian carcinomas". However, all present literatures took CA125 as the control in evaluating the value of HE4 in diagnosis, and so future studies should pay attention to setting of more scientific control, which is worth discussing.

This study found that although there was difference of HE4 albumen expression in tissues and serum between LGSG and HGSG, there was no difference at early stage FIGO (stage Ⅰ+Ⅱ), which indicated that the value of HE4 in early diagnosis of subtype ovarian carcinomas was still to be further studied.

In early diagnosis concerning subtype Ⅱ "small size ovarian carcinomas", we found that FIGO staging of LGSG featured stages Ⅰ-Ⅱ while of HGSG featured stages Ⅲ-Ⅳ. Pilot studies showed that FIGO staging could influence HE4 level, and the serum HE4 was higher at stages Ⅲ-Ⅳ than at stages Ⅰ-Ⅱ. Therefore, it is necessary to analyze HE4 level within the same FIGO, so as to eliminate the interference. However, when we analyzed within FIGO (Ⅰ+Istage Ⅰ), we found that in serum HE4 level there was no significant difference between LGSG and HGSG, which indicated that serum HE4 might be helpless in differential diagnosis of early stage subtype Ⅰ and subtype Ⅱ ovarian carcinomas. The reasons might be as follows: 1) HE4 is a secretory type albumen, and the mechanism for secretion into blood is still unclear. Accordingly, it was speculated that at stages Ⅰ and Ⅱ, the tumor vasculature had not been fully formed, and so release in blood was limited. 2) In cases of "early stage " by FIGO grading, the proportion with subtype Ⅱ cancer was low (15%), which was identical to biological characteristics of subtype Ⅱ cancer which progress rapidly[6]. The proportion of cases with subtype Ⅰ ovarian carcinomas was high while with subtype Ⅱ cancer was low, which was identical to the conclusion of present study. Obviously, this is not good for discussion on value of early diagnosis for subtype Ⅱ ovarian carcinomas. Therefore, at present, the value of HE4 for diagnosis of subtype Ⅱ tumor cannot be appraised.

Present literatures overestimated the value of HE4 for early diagnosis, and so it is necessary to analyze the rationality of the study designs and conclusions for these literatures. As a whole, early diagnosis for ovarian carcinomas is a great challenge, and it is difficult to use a single method to include early diagnosis of serum tumor markers. Locally, 1) The concept concerning basis on "dualistic pathogenesis model", and "early diagnosis" is unclear. Traditional early diagnosis is based on a concept that ovarian masses should be first formed for ovarian carcinomas, while according to dualistic pathogenesis model (dualistic model) of ovarian carcinomas, serous carcinoma is not originated from ovary itself, but from secondary planting of cells at fimbriae tubae. Therefore, when the tumor has violated the ovary, it has been metastatic carcinoma, not primary cancer. Accordingly, some scholars questioned the concept of "early diagnosis" for ovarian carcinomas, and proposed that "early diagnosis of ovarian carcinomas" should be replaced by "diagnosis of small size ovarian carcinomas[7]. From the view point of "dualistic pathogenesis model", early diagnosis should be from the tubal intraepithelial carcinoma. Now the tumor vasculature has not been fully formed, and so release in blood was limited. Therefore, the significance of serum tumor markers, including HE4 early diagnosis is still to be proved. How early should be for "early stage "? Most literatures took stage FIGO (Ⅰ+Ⅱ) as the standard of early stage, which is not necessarily reasonable. Although the concept of "small size ovarian carcinomas" has been put forward, but how small is small? There is still no specific quantitative criteria. 2) The "heterogeneity"of ovarian carcinomas and the key for early diagnosis was not reflected. The purpose of early diagnosis is early detection and treatment at early stage, by which the mortality may be reduced finally. From the view point of "heterogeneity" theory for ovarian carcinomas, subtype I ovarian carcinomas tumor grows slowly, mostly is limited to ovary, and only 25% of cases dead for ovarian carcinomas resulted from this tumor, while 75% resulted from subtype Ⅱ ovarian carcinomas, which is seldom limited once occurs, progresses rapidly, with poor prognosis, and is difficult to be found by early diagnosis. Therefore, the study on early diagnosis should focus on subtype Ⅱ cancer[7] (including HGSG). Present literatures failed to reflect this.

Therefore, our study, after a comprehensive analysis on the literatures, may only consider that HE4 is more sensitive and specific than CA125 in differentiating masses in terms of benign and malignancy, but the value of HE4 in early stage diagnosis of ovarian carcinomas cannot be directly indicated. The conclusions of present literatures, which overestimated the value of HE4 in early diagnosis of ovarian carcinomas, should be treated with discretion.

Our suggestion is that future studies should be based on heterogeneity of ovarian carcinomas, enlarge the sample size, and focus on evaluating the value of HE4 in early diagnosis of ovarian carcinomas.

Most cases at stage FIGO (Ⅰ+Ⅱ)of ovarian carcinomas incorporated in this study were of subtype Ⅰ serous carcinoma (76.1%), and rare cases were of subtype Ⅱ (13.9%), which conforms to present epidemiological data and biological characteristics of subtypes Ⅰ and Ⅱ ovarian carcinomas[7]. The inert biological behavior of subtype I ovarian carcinomas showed relative inert and progressive, and limited to ovary, and so when the disease was found, it was usually still at early stage by clinical staging, and it was easy to be found through intravagianal ultrasound and pelvioscopy, and so serum indexes are not of top priority[7]. The subtype Ⅰ ovarian carcinomas found in this study have pelvic masses to different extent, and the B type ultrasound indicated possibility of ovarian tumor, which indicated that, 1) The literatures overestimated the value of HE4 in early diagnosis, and maybe the deviation occurred because most incorporated cases at FIGO (Ⅰ+Ⅱ) were of subtype I tumor. 2) For control set for evaluating the true value of HE4 in diagnosis of subtype Ⅰ "small size ovarian carcinomas", the transvaginal ultrasonography might be more reasonable than CA125, which is lack of specificity. Recently, a prospective study[14] compared the risk of ovarian malignancy algorithm constructed based on serum HE4 and CA125 with transvaginal ultrasonography for diagnosis of ovarian carcinomas in value, and the results were that subjective diagnosis based on transvaginal ultrasonography was superior to risk of ovarian malignancy algorithm, which also indicated that whether HE4 is superior to traditional ultrasonography in diagnosis of subtype Ⅰ ovarian tumor is still to be further discussed.

In conclusion, ovarian carcinomas are of disease of "heterogeneity". Our study indicated that serum CA125 cannot distinguish subtype Ⅰ and subtype Ⅱ ovarian carcinomas, and so is not an ideal control for study of early diagnosis by HE4. serum HE4 level might have significance in differential diagnosis of subtype Ⅰ and subtype Ⅱ ovarian carcinomas. However, in early stage cases of FIGO (stages Ⅰ + Ⅱ), there was no difference of serum HE4 level between the two subtypes of ovarian carcinomas, and at the same time, at early stage, the proportion with subtype Ⅱ "small size ovarian carcinomas" was relative low, and so the value of serum HE4 for early diagnosis of subtype Ⅱ "small size ovarian carcinomas" was not sure. Our suggestion is that future studies should be based on heterogeneity of ovarian carcinomas, enlarge the sample size, and focus on evaluating the value of HE4 in early diagnosis of type Ⅱ ovarian carcinomas.

Conflict of interest statement

We declare that we have no conflict of interest.

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