Expression and significance of the protein and mRNA of metastasis suppressor gene Me491/cd63 and integrin α5 in ovarian cancer tissues

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Summary

Purpose: To investigate the expression and significance of the proteins and mRNA of metastasis suppressor gene Me491/cd63 and integrin α5 in ovarian cancer tissues. *Methods:* RT-PCR and in situ hybridization were used to detect the expression of the proteins and mRNA of ME491/CD63 and integrin α5 in normal ovarian tissues (Group I), ovarian benign tumor tissues (Group II), ovarian borderline tumor tissues (Group III) and ovarian cancer tissues (Group IV), and the correlation between the expression and the age of patient, degree of differentiation, lymphatic metastasis, stage and pathological type was analyzed. *Results:* There was a significant change in gene expression between the well and moderately differentiated tumors and poorly differentiated tumors. Gene expression in Groups III and IV was significantly weak, and significantly different from that in Group II and the early-stages of Groups III and IV. There was a significant difference in gene expression between each group. *Conclusion:* There is low expression of the proteins and mRNA of ME491/CD63 and integrin α5 in ovarian cancer. The lower the pathological differentiation is, the more significant the loss of expression is and the more likely metastasis is.

Key words: Ovarian cancer; ME491/CD63; Integrin α5; Metastasis.

Introduction

Infiltrative metastasis of tumor is a hard nut to crack in the field of oncology. Most malignant tumor patients die from complications caused by the widespread metastasis of tumors instead of primary diseases [1]. Ovarian cancer is the leading cause of death for gynecologic malignant tumor patients, which has special biological behavior and invasion sites and is inclined to present with multi-pathway and multi-region metastases. More than 80% of ovarian cancer patients die from infiltrative metastasis of the tumor [3]. In this research, RT-PCR and hybridization in situ (HIS) were used to detect the expression of the protein and mRNA of ME491/CD63 and integrin α5 in ovarian cancer tissues and to explore its clinical significance.

Materials and Methods

The patients visiting the Department of Obstetrics and Gynecology of the second affiliated hospital of China Medical University between June 2002 and June 2004 were included in this research. The 30 cases in the control group (Group I) were the normal ovarian tissues obtained during surgery (22-56 years old; mean 48.7). The ten cases in the ovarian benign tumor group (Group II) included five cases of serous cystadenoma, three cases of mucinous cystadenoma and two cases of other benign tumors (17-65 years old; mean 49.2). Six cases of ovarian borderline tumors (Group III) and 48 cases of ovarian cancers (Group IV) were also included. All the cases had been confirmed by postoperative pathological examinations (see Tables 1 and 2 for pathological type, degree of differentiation, pathological stage and lymphatic metastasis information). According to FIGO staging criteria (1988), there were seven cases in Stage I, nine cases in Stage II and 32 cases in Stages III and IV. The ages of the 48 ovarian cancer patients were between 21-64 years (mean 51.6).

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HIS detection

This method was used to detect the expression of the protein of ME491/CD63 and integrin α5. The primer sequence of human ME491/CD63 target gene was as follows: (The reagent kit used was the oligonucleotide probe for melanoma-associated antigen MLAL (CD63) labeled with digoxin. Product number: MK2325):

5'----GCGGT-GGAAG-GAGGA -ATCAA -ATGTG -TGAAG----3';

Product number: MK2390):

5'----GAGCC -TGTGG -AGTAC -AAGTC -CTTGC -AGTGG ----3';

Procedure: The test was performed according to the directions for the reagent kit (Wuhan Boster Biological Technology Co., Ltd.) and the method in reference [12]. 1) Routine dehydration and paraffin embedding methods were used. The thickness of the sections was 6-8 μm. 2) Polylysine was used to prepare the glass slides. 3) Routine deparaffinating was conducted on the sections. 4) The fragments of mRNA were exposed. Phosphate buffer (PBS) was used to rinse the sections three times (5 min each time). Distilled water was used to wash the sections one time. 5) After fixing: 1% paraform/0.1 mol/l PBS (pH 7.2-7.6) was used for fixation at room temperature for 10 min. Distilled water was used to rinse the sections three times. 6) Prehybridization: 20 μl prehybridization liquid was added to each section. 7) Hybridization: 20 μl hybridization liquid was added to each section. 8) Washing after hybridization. 9) Blocking solution was dripped onto the sections and the sections were placed at 37°C for 30 min. 10) Biotinylated mouse anti-digoxin antibody was added and the sections were placed at 37°C for 60 min or placed at room temperature for 120 min. PBS was used to rinse the sections four times (5 minutes each time). 11) Biotinylated peroxidase was added and the sections were placed at 37°C for 20 min or placed at room temperature for 30 min. 12) Diaminobenzidine was dripped onto the sections for color development and brazilein might be added for after staining if necessary. 13) Alcohol dehydration, xylene clearing and mounting were performed.

Determination of HIS results

The expression intensity of each gene was expressed with a histological score. The histological score was Σpi (i represented the staining intensity of cytoplasm). i = 0, 1, 2 and 3, respectively, indicated negative (no color), weakly positive (light brownish yellow), positive (brownish yellow) and strongly positive (deep brownish yellow). P represented the percentage of cells with the same staining intensity in all the cells counted; 1, 2, 3, 4 and 5, respectively, represented 0%-15%, 16%-50%, 51%-85%, 86%-95% and 96%-100% of the total number of cells. Image analysis was performed with the MPIAS-500 color image analysis system (Qingping Image Engineering Company of Tongji Medical University). Epithelial cells and interstitial cells were respectively detected and counted. Three fields were randomly selected in each section and the average value of histological score was used as the intensity of gene expression.

PCR detection

This method was used to detect the expression of ME491/CD63 and integrin α5 mRNA. Procedure: 1) Reagents: The reagent kits, CD63, integrin α5 and β-actin primers were all provided by Shanghai Bioasia Biotechnology Co., Ltd. 2) Extraction of total RNA in tissues: This step was performed according to the directions for the Trizol extraction reagent kit. An ultraviolet spectrophotometer was used to measure the purity of RNA and it was preserved in a –70°C refrigerator.

Sequencing of CD63 primer: (product 417bp) upstream 5'--ACC TGC TGT TCG GAT TTA-3', downstream 5'--TCA ACG CAT AGT GGA TGG-3'. β-actin was used as the internal reference (upstream primer sequence 5'--GAA ACT ACC TTC AAC TCC ATC-3', downstream primer sequence 5'--CTA GAA TTT GCG GTG GAC GAT GGA GGG GCC-3').

Primer sequence of integrin: (320 bp) upstream 5'-CAT TTC CGA GTC TGG GCC AA-3', downstream 5'-TGG AGG CTT GAG CTG AGC TT-3'. 3) Reverse transcription reaction: 2 μl RNA and 1 μl primer were added to 20 μl reverse transcription reaction system and the reaction was performed at 37°C. 4) PCR amplification: 3 μl cDNA, 0.05 μl upstream primer, 0.05 μl downstream primer and 0.2 μl heat resistant DNA-polymerase were added to PCR reaction system for amplification. 5) Electrophoresis: 1.5% agarose electrophoresis was performed on PCR products and PCR products were identified under ultraviolet transmissometer.

Determination of PCR results

Two percent agar gel electrophoresis was used to examine PCR products and an ultraviolet transmissometer was used to observe PCR products and take photos. The average absorbance (A value) of amplification band was measured after analysis system scan and the expression index (I) of mRNA of each gene was calculated. β-actin was used as an internal reference and the relative I value (CD63, integrin α5 gene) was A (CD63 or integrin α5)/A (β-actin). The loss rate was the percentage of cases with negative expression of various clinical pathological indices in the total cases.

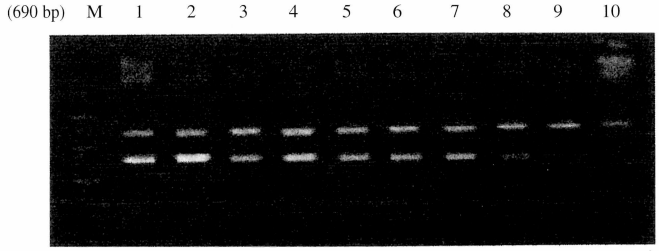
Statistical method

SPSS software was used for statistical analysis and the result was expressed as $x \pm s$. The Student's t-test, analysis of variance and correlation were used.

Results

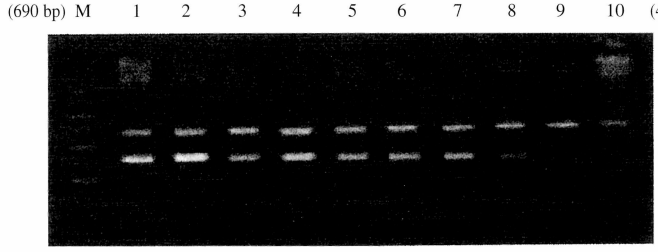
The expression of the mRNA of ME491/CD63 and integrin α5 in each group (Figures 1 and 2)

There was positive expression of these indices in both Group I (control group) and Group II (ovarian benign tumor group). The relative absorbances (I) were respectively, 1.658 ± 0.307 and 1.515 ± 0.299 for ME491/CD63 and 1.658 ± 0.307 and 1.515 ± 0.299 for integrin α5. The loss rates of both indices in Group III were 33.3% and the relative absorbance was 1.100 ± 0.469 for ME491/CD63 and 1.100 ± 0.469 for integrin α5. In Group IV the loss rate was 64.3% for ME491/CD63 and 68.8% for integrin α5 and the relative absorbance was 0.624 ± 0.402 for ME491/CD63 and 0.626 ± 0.402 for integrin α5. The X^2 value for comparison among groups was 16.615 and the F value was 14.606 ($p < 0.01$).



1, 2: Normal ovary; 3, 4: Benign tumor; 5, 6: Ovarian cancer (Stages I and II); 7, 8: Borderline ovarian tumor; 9, 10: Late-stage ovarian cancer (Stages III and IV).

Figure 1. — Electrophoresis results for the detection of ME491/CD63 mRNA of each group using RT-PCR.



1, 2: Normal ovary; 3, 4: Benign tumor; 5, 6: Ovarian cancer (Stages I and II); 7, 8: Borderline ovarian tumor; 9, 10: Late-stage ovarian cancer (Stages III and IV).

Figure 2. — Electrophoresis results for the detection of integrin α5 of each group using RT-PCR.

The expression of the mRNA of ME491/CD63 and integrin α5 in the ovarian cancer tissues with different clinical pathological indices (Table 1)

The X^2 value for the comparison among different age and pathological type groups was 0.028 and the F value was 0.094 ($p > 0.05$). The X^2 value for the comparison among the group with lymphatic metastasis, the group without lymphatic metastasis and the groups with different pathological differentiations was 3.136, and the F value was 17.365 ($p < 0.05$). The X^2 value for the comparison among the groups with different pathological stages was 17.865, and the F value was 16.946 ($p < 0.01$).

Table 1. — *Expression of the mRNA of ME491/CD63 and integrin α5 in ovarian cancer tissues with different clinical pathological indices.*

Clinical pathological indices	Number of cases	Integrin α 5 Loss rate (%)	Integrin α 5 I	ME491/CD63 Loss rate (%)	ME491/CD63 I
Age (years)					
≤ 60	29	66	0.7 ± 0.4	66	0.7 ± 0.4
> 60	19	74	0.6 ± 0.5*	63	0.7 ± 0.5*
Lymphatic metastasis					
Positive	5	5/5	0.5 ± 0.5	5/5	0.5 ± 0.5
Negative	7	1/7	0.7 ± 0.5**	2/7	0.7 ± 0.5**
Not examined	36	–	0.8 ± 0.5	–	0.8 ± 0.5
Pathological stage					
Stage I	7	0/7	0.9 ± 0.5	1/7	0.9 ± 0.5
Stage II	9	3/9	0.7 ± 0.4	2/9	0.7 ± 0.4
Stage III, IV	32	94	0.4 ± 0.4#	91	0.4 ± 0.4#
Pathological differentiation					
Well differentiated	17	53	0.7 ± 0.5	53	0.7 ± 0.5
Moderately differentiated	16	63	0.6 ± 0.5**	50	0.6 ± 0.5**
Poorly differentiated	15	93	0.6 ± 0.3	93	0.6 ± 0.3
Pathological type					
Serous	27	74	0.4 ± 0.3	70	0.4 ± 0.3
Mucous	5	2/5	0.6 ± 0.4	1/5	0.6 ± 0.4
Endometrioid	6	4/6	0.5 ± 0.3*	4/6	0.5 ± 0.3*
Others	10	70	0.5 ± 0.3	70	0.7 ± 0.5

Note: *X^2 0.028; F 0.094; $p > 0.05$; ** X^2 3.136; F 17.365; $p < 0.05$; # X^2 17.865: F 16.946; $p < 0.01$; – Not calculated.

Table 2. — *Expression of the proteins of integrin α5 and ME491/CD63 in the ovarian cancer tissues with different clinical pathological indices (number of cases).*

Clinical pathological indices	Number	Integrin α 5 Positive	Integrin α 5 Negative	ME491/CD63 Positive	ME491/CD63 Negative
Age (years)					
≤ 60	29	9	20	10	19
> 60	19	6	13	5	14##
Lymphatic metastasis					
Positive	5	0	5	0	5*
Negative	7	5	2	5	2
Not examined	36	–	–	–	–
Pathological stage					
Stage I	7	7	0	7	0
Stage II	9	6	3	6	3
Stage III, IV	32	2	30	2	30**
Pathological differentiation					
Well differentiated	17	8	9	7	10#
Moderately differentiated	16	7	9	6	10
Poorly differentiated	15	0	15	2	13
Pathological type					
Serous	27	7	20	7	20##
Mucous	5	3	2	4	1
Endometrioid	6	2	4	2	4
Others	10	3	7	2	8

Note: * r 0.33; $p > 0.05$; ** r 0.375; $p < 0.05$; # r 0.310; $p < 0.05$; ## $p > 0.05$.

The expression of the proteins of ME491/CD63 and integrin α5 in each group (Figures 3 and 4)

There was no negative expression of the proteins of ME491/CD63 and integrin α5 in the control group (Group I) and the ovarian benign tumor group (Group II). The loss rates of the expression of the two indices were 50% in the ovarian borderline tumor group (Group III). In the ovarian cancer group (Group IV), the expression of the proteins of ME491/CD63 and integrin α5 was positive in Stage I and the loss rate for Stage II was 33.3%. The loss rates for Stages III and IV were both 93.8%. There was significant difference between Groups I and II and Group III ($p < 0.05$) and between Groups I and II and Group IV ($p < 0.01$).

The expression of the proteins of ME491/CD63 and integrin α5 in the ovarian cancer tissues with different clinical pathological indices (Table 2)

The r value for the comparison among different age and pathological type groups was 0.632 ($p > 0.05$). The r value for the comparison between the group with lymphatic metastasis was 0.33 ($p < 0.05$). The r value for the comparison among the groups with different pathological differentiations was 0.310 ($p < 0.05$). The r value for the comparison among the groups with different pathological stages was 0.375 ($p < 0.05$).

Discussion

Significance of the expression of ME491/CD63 in ovarian cancer

ME491/CD63 is a member with tumor metastasis inhibiting effect of TM4SF family and is a lysosome membrane adhesion glycoprotein. CD63 genome DNA was used to transfect CD63 negative human melanoma cells and it was found that CD63 could inhibit the growth of human melanoma cells in nude mice, reduce hematogenous metastasis, and prevent the infiltration and metastasis of melanoma [5, 6]. The related studies showed CD63 could influence the movement, metastasis and growth of cells, CD63 could influence the growth of cells in vivo but could not influence the growth of cells in vitro, indicating CD63 might act as some kind of ligand receptor in vivo [7].

The experimental results showed there was expression of ME491/CD63 in each group. There was no significant difference in the comparison among Group I, Group II and Stage I of Group IV ($p > 0.05$). Similar to integrin α5, there was significant difference in the expression of ME491/CD63 between Group III and the above groups ($p < 0.05$). Borderline tumor presented with lymphatic metastasis early clinically and was resistant to chemotherapy. The intensity of ME491/CD63 expression changed

with the change of the stage of cancer. The gene expression of ME491/CD63 gradually decreased in Stages II, III and IV, and there was a significant difference between the above groups ($p < 0.01$). The decrease of the expression of ME491/CD63 conduced to the detachment of tumor cells after contacting the matrix, facilitated the infiltration and metastasis of tumor cells via ECM, and might take part in the infiltration and metastasis of tumors resulting in manifestations of consumption. There was no significant difference in the expression of ME491/CD63 between the different age groups and the groups with different pathological types of ovarian cancer ($p > 0.05$). On one hand, these two factors had no influence on its expression. On the other hand, the number of cases, antibody titer and the sensitivity of methods had an influence on its expression. The expression was negative in the cases with lymphatic metastasis. The negative expression rate was 28.57% in the tumor tissues without lymphatic metastasis. There was a significant difference between the group with lymphatic metastasis and the group without lymphatic metastasis ($p < 0.05$). Since the dissection of the lymph nodes in the pelvic cavity was not necessary for ovarian cancer in late stage, there was a defect in the discussion on the lymphatic metastasis factor. There was no significant difference between the well-differentiated group and the moderately differentiated group ($p > 0.01$). However, there was significant difference between the well-differentiated group and the poorly differentiated group and between the moderately differentiated group and the poorly differentiated group ($p < 0.01$). Therefore, the poorer the differentiation was, the higher the malignancy was and the stronger the down-regulation of ME491/CD63 and the trend of loss, infiltration and metastasis were.

Significance of the expression of integrin α5 and its relation to the generation and development of ovarian cancer

Integrin is an integral protein in membranes and a heterodimer complex composed of α subunits and β subunits via a non-covalent bond. As a member of the integrin family, integrin α5 is a kind of fiber-binding receptor, which participates in the signal transmission of cells and regulates the adhesion and migration of cells. Changes in the adhesion and migration of cells play an important role in the formation, infiltration and dissemination of tumors. The adhesion of cells to the extracellular matrix was realized through receptors, among which most were integrin receptors. The adhesion receptors on the surface of tumor cells (integrin) could only realize the infiltration and metastasis of cells after adhesion to the constituents of the extracellular matrix [2, 4].

The experimental results showed there was expression of integrin α5 in each group, and there was no significant difference among Group I, Group II and Stage I of Group IV ($p > 0.05$). However, there was a significant difference between Group III and the above groups ($p < 0.05$). The sample size should be increased in order to explain why metastasis is more inclined to occur in borderline tumors. There was no significant difference between Group III and Stage II of Group IV, but there was a significant difference between Group III and Stages III and IV of Group IV ($p < 0.01$). However, there was a change in the expression of integrin α5 with the development of cancer and increase of stage. The expression of integrin α5 gradually decreased in Stages II, III and IV, which was significantly different from the above groups ($p < 0.01$). It was suggested in the study that the decreased expression of integrin α5 conduced to the detachment of tumor cells after contacting the matrix, facilitating the infiltration and metastasis of tumor cells via ECM, perhaps taking part in the infiltration and metastasis of tumors thus resulting in manifestations of consumption. However, a contradictory conclusion has been obtained in related research on lung cancers, that is, the high expression of integrin α5 indicated the high incidence of lung cancer and poor prognosis [5]. There was no significant difference in the expression of integrin α5 between the different age groups and the groups with different pathological types. The expression of integrinα5 was negative in the cases with lymphatic metastasis. The negative expression rate was 42.86% in the tumor tissues without lymphatic metastasis. There was a significant difference between the group with lymphatic metastasis and the group without lymphatic metastasis ($p < 0.05$). There was no significant difference between the well differentiated group and the moderately differentiated group ($p > 0.01$). However, there was a significant difference between the well-differentiated group and the poorly differentiated group and between the moderately differentiated group and the poorly differentiated group ($p < 0.01$). Therefore, the poorer the differentiation was, the higher the malignancy was, the lower the expression of integrin α5 was and the stronger the trend of infiltration and metastasis was.

The mutual identification between integrin α5 and its ligands is closely related to the generation and development of tumors. There was no significant difference in the expression of ME491/CD63 between Stage I and Stage II, but there was significant difference in the expression of integrin α5 between Stage I and Stage II. This may be the cause of their synergic and independent actions in the infiltration and metastasis of ovarian cancer.

Action of E491/CD63 and integrin α5 in the metastasis of ovarian cancer

The trends of changes of E491/CD63 and integrin α5 were consistent in this experiment, indicating their simultaneous existence and actions in inhibiting metastases of ovarian cancer or the formation of some kind of relation between them [7]. It has been proven by related experiments that the reaction of ME491/CD63 to some kind of integrin is highly specific, the subunit of integrin can specifically bind with ME491/CD63 protein complex, regulate the adhesion of cells, and regulate and control the growth and metastasis of cells [8]. The combination of these two protein components results in the regulation of the growth and metastasis of tumor cells by both integrin family adhesion receptor and ME491/CD63 protein,

indicating ME491/CD82 protein can regulate the adhesion function of integrin α5 [9].

It has also been found that ME491/CD63 protein and integrin α5 can bind with a specific enzyme which participates in the synthesis of phosphatidylinositol and leads to the generation of 4, 5-PIP2; 4, 5-PIP2 is a confirmed regulator for the structure of cytoskeleton and is concerned with the extension and transport of cells. The adhesion reactions mediated by integrin activate small GTP structural protein Rho, which may participate in the generation of PIP2 [10, 11]. Therefore, it is deduced that the integrin α5/Kail/CD63 protein complex plays a key role in the regulation of the Rho dependent function. ME491/CD63 protein may influence the adhesion of cells and integrin-mediated cell metastasis by regulating the functions of integrin.

Conclusion

There is low expression of the proteins and mRNA of ME491/CD63 and integrin α5 in ovarian cancer. The lower the pathological differentiation is, the more significant the loss of expression is and the more likely metastasis is.

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