Overexpression of EFEMP1 Correlates with Tumor Progression and Poor Prognosis in Human Ovarian Carcinoma

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Abstract

Objective: This study was to explore the role of EFEMP1 in ovarian tumor progression and its relationship with prognosis of ovarian carcinoma.

Methods: EFEMP1 mRNA and protein expressions in normal ovarian tissue, ovarian tumor, high invasive subclones and low invasive subclones were evaluated by immunohistochemistry and real time RT-PCR. Serum EFEMP1 levels in patients with ovarian tumor were measured by ELISA assay. To assess the angiogenic properties of EFEMP1, VEGF and tumor microvessel density were analyzed in ovarian carcinoma by immunohistochemistry.

Results: EFEMP1 expression was up-regulated in ovarian carcinoma, positively correlated with MVD and VEGF, and its overexpression and high serum levels were significantly associated with high stage, low differentiation, lymph node metastasis and poor prognosis of ovarian cancer. EFEMP1 expression was also found to be over-expressed in the highly invasive subclones compared with the low invasive subclones.

Conclusion: EFEMP1 is a newly identified gene over-expressed in ovarian cancer, associated with poor clinicopathologic features and promotes angiogenesis. This study shows that EFEMP1 may serve as a new prognostic factor and a therapeutic target for patients with ovarian cancer in the future.

Introduction

Ovarian cancer is one of the most aggressive and heterogeneous cancer types in women and one of the leading causes of gynaecological deaths [1,2]. Its high mortality is attributable to the fact that the majority of ovarian cancer patients are diagnosed at advanced stages when conventional therapy is less effective [3]. Although substantial advances have been made in ovarian cancer research, the overall 5-year survival rate is still less than 30% [4]. Tumor recurrence and metastasis are considered the major reasons for poor clinical outcome and cancer deaths [5]. Therefore, studying the mechanism of tumor invasion and metastasis will provide further insights into the development and progression of ovarian cancer. In recent years, many biomarkers have been investigated which are involved in the progression of ovarian cancer [6]. But few studies have been done to assess the functions of EFEMP1 in ovarian cancer development.

EFEMP1 (epidermal growth factor–containing fibulin-like extracellular matrix protein 1, fibulin-3) is a member of the fibulin family of extracellular glycoproteins, which are characterized with a fibulin-type C-terminal domain preceded by tandem calcium binding epidermal growth factor (EGF)-like modules [7]. Fibulins have been shown to modulate cell morphology, growth, adhesion and motility, closely related to a wide variety of cancer development [8]. Overexpressions of fibulin-1 in ovarian cancer and breast cancer have been found to demonstrate oncogenic activity, during these progressions estrogen play important roles [9–11]. However in the development of hepatocellular carcinoma, gastric cancer and prostate cancer, fibulin-1 demonstrated tumor-suppressive activity, suppressing tumor growth, enhancing cell apoptosis and inhibiting tumor angiogenesis [12–15]. As a tumor suppressor gene, Fibulin-2 inhibited tumor growth, invasion and angiogenesis in hepatocellular carcinoma and breast cancer [16,17]. In the majority of the development of cancers, Fibulin-5 was widely considered to be associated with the suppression of tumor formation through its control of cell proliferation, motility and angiogenic sprouting [18–20].

Paradoxically, EFEMP1 (fibulin-3) can also demonstrate either tumor-suppressive or oncogenic behavior tied to tissue-specific expression. In the research of pancreatic adenocarcinoma, cervical cancer and glioma [21–24], increased expression of EFEMP1 has
been reported; EFEMP1 plays a role in metastasis and development, and thus links it to poor prognosis. In contrast, a potential cancer-suppressing function of EFEMP1 was found in the study of nasopharyngeal carcinomas, sporadic breast cancer, glioblastoma multiforme, and non-small cell lung cancer (NSCLC). Fibulin-3 was associated with tumour progression and inhibited cell migration and invasion [25–28]. But till now, few researches have been made about the relationship between EFEMP1 and ovarian cancer.

The purpose of this study was to assess whether EFEMP1 expression was associated with the prognosis of ovarian cancer, and further to investigate the relation of EFEMP1 expression to angiogenesis.

Figure 1. Expression of EFEMP1 in human ovarian tissues. (A) The epithelial cells of normal human ovarian. (B) the stroma of normal human ovarian. (CD) Benign ovarian tumor, (E) High differentiation of ovarian carcinoma, (F) Medium differentiation of ovarian carcinoma, (G) Low differentiation of ovarian carcinoma. (Magnification ×200). doi:10.1371/journal.pone.0078783.g001
Materials and Methods

Cell lines

The highly invasive subclones (S1, A1) and the low invasive subclones (S21, A19) were isolated from the human ovarian cancer cell lines SKOV3 and 3AO with the limited dilution method. Then, the cell electrophoretic mobility (EPM) of each clone was measured to study charge property using microcapillary electrophoresis (microCE) chips according to Omasu’s methods [29]. Finally, MTT assay, Colony formation assay in soft agar, Matrigel invasion assay, Cell migration assay and Tumor xenografts in nude mice were made to confirm that they had high and low metastatic potentials respectively [30,31]. Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics (Gibco BRL, Rockville, MD).

Tissue Specimens

A total of 260 human ovarian tissue specimens were used for this study. Two hundred and twenty (220) epithelial ovarian tumors were obtained from the Department of Gynecology and Obstetrics, Shandong Provincial Hospital between 2005 and 2008. The ovarian tumor specimens included 60 benign ovarian tumors and 160 epithelial ovarian carcinomas (with 58 serous cystadenocarcinoma, 56 mucinous cystadenocarcinoma and 46 endometrioid carcinoma). All of the ovarian cancer patients were clinically staged according to the FIGO staging system [with 74 low stage tumors (FIGO stages I and II) and 86 high stage tumors (FIGO stages III and IV)]. None of the ovarian cancer patients received preoperative radiation or chemotherapy. Forty (40) normal ovary tissue specimens were obtained from the Department of Gynecology and Obstetrics, Shandong Provincial Hospital. The study was approved by the Institutional Medical Ethics Committee of Shandong University.

Blood samples

Blood samples were accordingly obtained with the written informed consent from the same 160 women with epithelial ovarian cancer (with 58 serous cystadenocarcinoma, 56 mucinous cystadenocarcinoma and 46 endometrioid carcinoma) and from the same 60 women with benign ovarian tumor at the Department of Gynecology and Obstetrics, Shandong Provincial Hospital between 2005 and 2008. None of the ovarian cancer patients

Figure 2. Expression of EFEMP1 in human ovarian tissues with higher-magnification. (A) The epithelial cells of normal human ovarian, (B) the stroma of normal human ovarian, (C) Benign ovarian tumor, (D) High differentiation of ovarian carcinoma, (E) Medium differentiation of ovarian carcinoma, (F) Low differentiation of ovarian carcinoma. (Magnification ×400).

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received preoperative radiation or chemotherapy. Sixty control blood samples were obtained from age-matched examinees receiving health checks at Shandong Provincial Hospital and showing no history of disease and no abnormalities in laboratory examinations. The study was approved by the Institutional Medical Ethics Committee of Shandong University.

Enzyme-linked immunosorbent assay

Levels of EFEMP1 in serum samples were measured with sandwich enzyme-linked immunosorbent assay (ELISA) using human EFEMP1 ELISA assay kits (Immuno-Biological Laboratories, Japan). Serum was diluted with EIA buffer (1% BSA, 0.05% Tween 20 in phosphate buffer) and incubated for 2 hour at 37°C. After 4 washes with EIA buffer, horse radish peroxidase-conjugated antibodies were added and incubated for 30 minutes at 4°C. After 4 washes, 100 µL of tetramethyl benzidine solution was added and incubated for 30 minutes at room temperature. The reaction was stopped with 100 µL of 1 N sulfuric acid and measured by ELISA reader at 450 nm.

Immunohistochemistry (IHC)

According to standard streptavidin-biotin-peroxidase complex procedures, IHC was performed on formalin-fixed, paraffin-embedded sections (5 µm thick) and cell slides fixed in 4% paraformaldehyde. Briefly, after dewaxing, rehydration, and antigen retrieval, the sections were incubated with mouse anti-human EFEMP1 monoclonal antibody (sc-33722, Santa Cruz Biotechnology, Inc.) diluted 1:50 in PBS. Human cervical cancer paraffin-embedded sections (EFEMP1 positive) were used as positive controls. A negative control was obtained by replacing the primary antibody with normal mouse immunoglobulin (IgG). Positive expression of EFEMP1 protein was defined as the presence of brown granules in the cytoplasm.

Immunohistochemistry (IHC) analysis

A semiquantitative scoring system based on intensity of staining and distribution of positive cells was used to evaluate EFEMP1 expression. The intensity of EFEMP1 positive staining ranged from 0 to 3 (negative = 0, weak = 1, moderate = 2, or strong = 3) and the percentage of positively stained cells was scored as 0 (0%).

Figure 3. Positive control and negative control of immunohistochemistry (IHC) analysis. Positive control (EFEMP1 positive) (A) Human cervical cancer paraffin-embedded sections. Negative control (B) normal human ovarian tissue, (C) Benign ovarian tumor, (D) High differentiation of ovarian carcinoma, (E) Medium differentiation of ovarian carcinoma, (F) Low differentiation of ovarian carcinoma. (Magnification ×200).

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1 (1 to 25%), 2 (26 to 50%), 3 (51 to 75%), and 4 (76 to 100%). The sum of the intensity and percentage score was used as the final staining score (0 to 7). The sum-indexes (−), (+), (++), and (+++) indicated final staining score of 0, 1–3, 4–5, and 6–7, respectively. For statistical analysis, sum-indexes (−) and (+) were defined as low EFEMP1 expression, while sum-indexes (+++) and (+++) were defined as high EFEMP1 expression. Each section was independently scored by two pathologists. If an inconsistency occurred, a third pathologist was consulted to achieve consensus.

Figure 4. EFEMP1 mRNA expression and Kaplan-Meier analysis. (A) EFEMP1 mRNA expression. High EFEMP1 mRNA expression was also associated with low differentiation, high stage and positive lymph node status of ovarian carcinomas. (B) Kaplan-Meier analysis. Patients with high EFEMP1 expression (blue line) had a much worse prognosis than those with low EFEMP1 expression (green line). *P < 0.05 versus control. doi:10.1371/journal.pone.0078783.g004

Microvessel assessment

The microvascular density (MVD) assessment was evaluated by CD34 immunohistochemical staining of tumor vessels. Any immune-positive single endothelial cell or endothelial cell clusters and microvessels in the tumor was considered to be an individual vessel and counted, as described by Weidner et al. [32]. Peritumoral vascularity, vascularity in areas of necrosis and vessels with thick muscle wall or in a diameter larger than eight erythrocytes, was excluded from the count. The sections were scanned at low power (100×) to select the most vascularized (hot-
sm10 primers were used: pathologic data. Observers who had no knowledge of the corresponding clinico-calculated as MVD. All counts were made by three independent
Statistical analysis
Real-time quantitative RT-PCR (q-RT-PCR)
Total RNA was extracted using Trizol reagent (Invitrogen) and reversed transcribed. Quantitative real-time PCR analysis was performed using ABI PRISM 7500 Real-Time PCR System (Applied Biosystems). Each well (20 μl reaction volume) contained 10 μl Power SYBR Green PCR master mix (Applied Biosystems), 1 μl of each primer (5 μmol/l) and 1 ul template. The following primers were used:
EFEMP1 5'-ACCCCTCCACCCGATCCA-3'
5'-TCTGCTCTCAGTTGTGGGTCC-3'
β-actin 5'-CCACGAAAATACCTTTCAACTTCA-3'
5'-GTGATCTCCTTCATCGATCTGC-3'
Statistical analysis
IHC data were analyzed using chi-square test. Measurement data were expressed as the mean ± SE. For comparison of means between two groups, a two-tailed t-test was used and for comparison of means among three groups, one-way ANOVA was used. Survival curve was calculated using the Kaplan-Meier method and the log-rank test. Correlation between the expression of EFEMP1 and VEGF and MVD were analyzed with Pearson correlation coefficient. Statistical analysis was performed using SPSS software version 13.0. P-value<0.05 was considered statistically significant.

Table 1. Protein expression of EFEMP1 in human ovarian tissues.

| Pathology type | EFEMP1 low (−/−) | EFEMP1 high (++/+++ | χ² | P |
|----------------|------------------|-------------------|----|----|
| Normal         | 40 38 95%        | 2 5%              | 66.01 | <0.01 |
| Benign         | 60 42 70%        | 18 30%            |      |     |
| Carcinoma      | 160 49 30.6%     | 111 69.4%         |      |     |
| Pathology type |                  | 0.236 0.889       |     |     |
| serous cystadenocarcinoma | 58 19 32.8%     | 39 67.2%        |      |     |
| mucinous cystadenocarcinoma | 56 16 28.6%     | 40 71.4%        |      |     |
| endometrioid carcinoma | 46 14 30.4%     | 32 69.6%        |      |     |
| Cell differentiation | 26.921 <0.01    |                  |     |     |
| High and Medium | 88 42 47.7%     | 46 52.3%        |      |     |
| Low            | 72 7 9.7%       | 65 90.3%         |      |     |
| Tumor stage    | 27.837 <0.01    |                  |     |     |
| Low stage      | 74 38 51.4%     | 36 48.6%         |      |     |
| High stage     | 86 11 12.8%     | 75 87.2%         |      |     |
| Nodal status   | 35.752 <0.01    |                  |     |     |
| Positive       | 83 8 9.6%      | 75 90.4%         |      |     |
| Negative       | 77 41 53.2%    | 36 46.8%         |      |     |

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Table 2. mRNA expression of EFEMP1 in human ovarian tissues.

| Pathology type | EFEMP1 mRNA | P |
|----------------|-------------|---|
| Control        | 40 0.0087±0.0053 |     |
| Benign         | 60 0.0079±0.0068 | >0.05* |
| Carcinoma      | 160 0.0738±0.0095 | <0.05** |
| Pathology type |             | >0.05 |
| serous cystadenocarcinoma | 58 0.0684±0.0103 |     |
| mucinous cystadenocarcinoma | 56 0.0849±0.0136 |     |
| endometrioid carcinoma | 46 0.0741±0.0181 |     |
| Cell differentiation |             | <0.05 |
| High and Medium | 88 0.0256±0.0097 |     |
| Low            | 72 0.0869±0.0127 |     |
| Tumor stage    |             | <0.05 |
| Low stage      | 74 0.0357±0.0083 |     |
| High stage     | 86 0.0851±0.0138 |     |
| Nodal status   |             | <0.05 |
| Positive       | 83 0.0732±0.0109 |     |
| Negative       | 77 0.0254±0.0067 |     |

Note:
*Benign ovarian tumor compared with healthy control, P>0.05;
**Ovarian carcinoma compared with healthy control and benign ovarian tumor, P<0.05.
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Results
Expressions of EFEMP1 in human ovarian tissues
To evaluate the mRNA and protein expressions of EFEMP1 in ovarian tissues, we detected 260 human ovarian tissue specimens, including 40 normal human ovaries, 60 benign ovarian tumors and 160 epithelial ovarian carcinomas by immunohistochemistry and real time RT-PCR. As shown in Figure 1 and 2, in normal human ovarian surface epithelial cells, EFEMP1 protein expression was very low (Fig. 1A and Fig. 2A), and in the ovarian stroma, EFEMP1 protein expression was mainly focused around the vasculatures (Fig. 1B and Fig. 2B). However in most ovarian carcinomas, the immunoreactivity was high, and high EFEMP1 protein expression was found in the cytoplasm of ovarian cancer cells (Fig. 1EFG and Fig. 2DEF). To determine the specificity of the antibody, the negative control and positive control was shown in figure 3. Moreover, high EFEMP1 protein expression was associated with low differentiation, high stage and positive lymph node status of ovarian carcinomas (Table 1). Similarly results were also found in real time RT-PCR experiment, EFEMP1 mRNA expression was also very low in normal ovarian tissues and benign ovarian tumors, and significantly enhanced in ovarian carcinoma. Moreover, high EFEMP1 mRNA expression was also associated with low differentiation, high stage and positive lymph node status of ovarian carcinomas (Table 2 and Figure 4A). To evaluate the prognostic value of EFEMP1 in ovarian cancer, we performed survival analysis using Kaplan-Meier analysis. The result showed that patients with high EFEMP1 expression had a much worse prognosis than those with low EFEMP1 expression (log rank, P<0.01; Figure 4B).
Different expression of EFEMP1 in the highly invasive subclone and low invasive subclone

The highly invasive subclone (S1 and A1) and the low invasive subclone (S21 and A19) were derived from the SKOV3 and 3AO human ovarian cancer cell line, by limited dilution method. As shown in Figure 5, EFEMP1 protein and mRNA expressions were very higher in highly invasive subclone (S1 and A1), compared to the low invasive subclone (S21 and A19).

Serum levels of EFEMP1 in human ovarian tumor and healthy control

As shown in Table 3 and Figure 6A, the serum EFEMP1 level of ovarian carcinoma was much higher than that of healthy control and benign ovarian tumor ($P<0.05$). No significant difference was found between healthy control and benign ovarian tumor ($P>0.05$). Moreover, high serum levels of EFEMP1 were associated with low differentiation, high stage and positive lymph
node status of ovarian carcinomas ($P<0.05$). There were no significant differences among different pathology types of ovarian carcinoma ($P>0.05$).

**Relationship between EFEMP1 and VEGF and MVD**

Figure 6BCDE shows the representative immunohistochemical staining of VEGF and CD34. The immunohistochemical expressions of VEGF and EFEMP1 were evaluated with semiquantitative scoring. Angiogenesis is the process of formation of new microvessels from the preexisting vasculature. Vascular endothelial growth factor (VEGF) is considered as the most potent candidate for the induction of angiogenesis in tumor growth. We want to know whether EFEMP1 is related to angiogenesis, so the Pearson correlation coefficient was calculated to assess the correlation between EFEMP1 and MVD, and the results showed a positive correlation, with high statistical significance (Fig. 7A, $r = 0.389$, $P<0.01$). Accordingly, by Pearson correlation coefficient, the correlation between EFEMP1 and VEGF also revealed direct relation with high statistical significance (Fig. 7B, $r = 0.243$, $P<0.01$).

**Discussion**

In the present study, we have demonstrated for the first time that the expression of EFEMP1 is associated with bad clini-
The Relationship between EFEMP1 and Ovarian Cancer

Table 3. Serum levels of EFEMP1 in patients with ovarian tumor.

| Pathology type                  | N  | EFEMP1 (ng/ml) | P      |
|---------------------------------|----|----------------|--------|
| Control                         | 60 | 126.47±15.83   |        |
| Benign                          | 60 | 132.76±18.57   | >0.05  |
| Carcinoma                       | 160| 319.51±21.65   | <0.05**|
| Pathology type                  |    |                |        |
| Serous cystadenocarcinoma       | 58 | 287.69±18.81   | >0.05  |
| Mucinous cystadenocarcinoma     | 56 | 274.53±22.74   | >0.05  |
| Endometrioid carcinoma          | 46 | 268.79±18.33   |        |
| Cell differentiation            |    |                | <0.05  |
| High and medium                 | 88 | 147.29±13.88   |        |
| Low                             | 72 | 304.37±20.26   |        |
| Tumor stage                     |    |                | <0.05  |
| Low stage                       | 74 | 134.73±19.54   |        |
| High stage                      | 86 | 335.81±22.69   |        |
| Nodal status                    |    |                | <0.05  |
| Positive                        | 83 | 325.49±23.57   |        |
| Negative                        | 77 | 129.73±16.44   |        |

Note:
*Benign ovarian tumor compared with healthy control, P>0.05;
**Ovarian carcinoma compared with healthy control and benign ovarian tumor, P<0.05.

Figure 7. Pearson correlations analysis of EFEMP1 expression with MVD and VEGF. The expression of EFEMP1 positively correlated with MVD (A) and VEGF (B). *P<0.05 versus control.

cell or a cervical cancer cell line, the tumors with EFEMP1 overexpression showed a faster growth rate and had a higher level of VEGF expression and microvascular density [25]. In contrast to our results, EFEMP1 was found to exert antiangiogenesis effect. Albig et al. discovered Fibulin-3 as novel antagonists of endothelial cell activities capable of reducing tumor angiogenesis and, consequently, tumor growth in vivo [33]. Such disparity may be due to the fact that tumor microenvironment influences the tumor genes to promote angiogenesis and metastasis [34]. Of cause, further researches need to be done in the future, including cell transfection experiment, chorioallantoic membrane (CAM) assay and tumor xenografts in nude mice assay to confirm our result.

High serum levels of EFEMP1 were also found in ovarian carcinoma rather than in healthy control and benign ovarian tumor, and associated with low differentiation, high stage and positive lymph node status of ovarian carcinomas. This discovery may aid in determining the diagnosis and prognosis of ovarian carcinoma. Similar result was found in pleural mesothelioma, the plasma fibulin-3 level was significantly elevated in patients with mesothelioma [35]. New biomarker can help to detect ovarian carcinoma at an earlier stage and to individualize treatment strategies.

In conclusion, EFEMP1 is a newly identified gene overexpressed in ovarian cancer, associated with poor prognosis and promotes angiogenesis. Serum levels of EFEMP1 may be helpful to early diagnosis and prognosis judgment. EFEMP1 may serve as a new prognostic factor and a therapeutic target for patients with ovarian cancer in the future.
**Author Contributions**

Conceived and designed the experiments: JC YZ. Performed the experiments: JC JZ DW XL. Analyzed the data: YZ DW. Contributed reagents/materials/analysis tools: JC JZ YZ. Wrote the paper: JC JZ.

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