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Up-regulation of stromal versican expression in advanced stage serous ovarian cancer

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Abstract

Objective—The purpose of this study is to examine the role of versican (VCAN) in advanced stage serous ovarian cancer by investigating its expression, its function, and its correlation with clinical outcomes.

Methods—Microarray analysis was performed on RNA isolated from tumor and stromal components of advanced stage serous ovarian cancer and normal ovarian epithelial tissue to identify genes up-regulated in ovarian tumor stroma. Validation studies using immunohistochemistry and quantitative real-time PCR (Q-RT-PCR) was performed on one of the up-regulated genes, VCAN. Immunolocalization of VCAN (n=111) and CD31 (n= 56) were done on serous ovarian tumors. CD31 staining was performed to examine microvessel density (MVD). Q-RT-PCR was performed on 65 samples to evaluate the differential expression of VCAN isoforms. Cell proliferation and invasion assays were performed to examine how V1-treated ovarian cancer cell lines and an endothelial cell line would differ from controls. Univariate survival analyses were done with VCAN expression. Correlation analysis was done with CD31, platinum resistance, and clinical data.

Results—Validation studies using Q-RT-PCR and immunohistochemistry showed significantly higher VCAN V1 isoform expression in ovarian cancer stroma compared with normal ovarian

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Conflict of Interest statement

The authors declare that there are no conflicts of interest.

stroma and ovarian cancer cells. Correlation studies showed stromal VCAN expression was associated with poorer overall and progression free survival, platinum resistance, and increased MVD. VCAN-treated ovarian cancer and endothelial cells showed increased invasion potential.

Conclusions—VCAN overexpression is associated with increased MVD and invasion potential, which may lead to poorer overall and progression free survival and platinum resistance.

INTRODUCTION

Epithelial ovarian cancer accounts for more deaths annually than all other gynecologic cancers combined [1]. Although early stage disease has 5-year survival that is near 95%, the majority of cases are diagnosed at an advanced stage [2]. With cytoreductive surgery and platinum and taxane-based chemotherapy, greater than half of advanced cases will achieve remission; but, most will experience recurrence and ultimately succumb from their disease [2]. Thus, biological therapies, which will complement standard surgery and chemotherapy, are sought to improve the survival in advanced stage epithelial ovarian cancer.

The extracellular matrix (ECM) is a three-dimensional structure which regulates cell migration, differentiation, and proliferation [3]. Modifications to the ECM composition during tumor development may be crucial for tumor initiation and progression [3]. The process of cancer cell invasion, metastasis, and growth likely involves alterations in cellular attachment to ECM, local proteolysis of the basement membrane, and migration through stroma to gain access to the circulation [4,5]. A decrease in the adhesive ability of tumor cells at the invasive foci has been noted for a number of human cancers [6,7]. Proteoglycans may play a role in the cell-ECM adhesion interactions during cancer progression and may be used as prognostic markers and therapeutic targets for the treatment of the disease [8-10].

Previous studies have shown that using the microdissected epithelial component of tumor specimens in large-scale transcription profiling can identify differentially expressed genes and molecular signatures in ovarian cancer [11]. Potential diagnostic and prognostic markers for the disease have been identified [11]. In this study, we discovered a gene signature for the microdissected fibroblastic component of the stroma by comparing the transcriptome profiles generated from the fibroblastic stromal and epithelial components of cancer tissue and the fibroblastic stromal component of normal ovaries. We further undertook the study to validate the differential expression of a secretory protein called versican (VCAN) and to investigate its functional roles and clinical significance.

MATERIALS AND METHODS

Study Patients and Tissue Samples

Paraffin-embedded and frozen tissues were collected and archived from patients undergoing surgery at Brigham and Women's Hospital (Boston, MA) between 1990 and 2006. All ovarian cancer tissues were the serous subtype, high-grade, and International Federation of Gynecology and Obstetrics (FIGO) stage IIIB-IV. All samples were collected from primary surgeries, and tumor tissues were processed with standard pathology methods. Clinical data including age, cytoreduction status (optimal vs. suboptimal), chemotherapies utilized, and overall survival were abstracted from the medical record. Optimal surgical cytoreduction was defined as residual tumor ≤ 1 cm in diameter. The duration of overall survival was measured from the date of diagnosis to death or censored at the date of last follow-up. All patient-derived specimens were collected under protocols approved by the institutional review board.

Microdissection, RNA isolation, amplification and hybridization, and Microarray analysis

Microdissection and RNA isolation were performed as previously described [11]. Thirteen stromal and 16 epithelial components from 7 μ m frozen sections of advanced stage serous ovarian cancer, and 10 stromal components of normal ovaries obtained from patients with benign gynecologic diseases were microdissected using a LMD laser microdissecting microscope (Leica, Wetzlar, Germany). RNA was isolated immediately and was extracted and purified using the RNeasy Micro kit (Qiagen, Valencia, CA). All purified total RNA specimens were quantified and checked for quality with a Bioanalyzer 2100 system (Agilent, Palo Alto, CA). Total RNA amplification and hybridization were performed as previously described [11]. Global normalization, quality control screening, and collation were performed as previously described [11]. Normalized data were uploaded into the NCI Microarray Analysis Database for quality-control screening and collation. BRB ArrayTools (version 3.5.0) software was used to filter the array data and complete the statistical analysis. Only those probe sets present in greater than 50% of the arrays and displaying a variance in the top 50th percentile were evaluated. Differentially expressed genes were identified using a multivariate permutation t-test ($P < 0.001$). A total of 2000 permutations were completed to identify a list of probe sets containing fewer than 5% false positives at a confidence of 90%.

Cell Lines

A total of 10 serous ovarian cancer cell lines were used (ALST, CAOV3, DOV3, OVCA420, OVCA429, OVCA432, OVCA433, SKOV3, SKOV3-luc, and OVCA3). Normal human ovarian surface epithelium (HOSE) cells were obtained from fresh ovarian scrapings during surgery for benign non-ovarian conditions. Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza Walkerville Inc. (Walkersville, MD). Chinese hamster ovary (CHO K1) and CHO K1 cells overexpressing recombinant V1 VCAN (CHO V1) were kindly provided by Professor R LeBaron. The CHO K1 and CHO V1 were maintained in α -MEM nucleoside-free medium (Invitrogen, Carlsbad, CA) as described previously [12].

Immunohistochemistry

Seven micron-thick sections from 111 paraffin-embedded tissue sections of advanced stage serous ovarian tumors and ten normal ovaries were placed onto Superfrost Plus glass slides. (Fisher Scientific, Pittsburgh, PA) Immunostaining was performed using the Zymed PicTure™-MAZ Polymer Detection Kit (Invitrogen, Carlsbad, CA). Immunostaining was then performed by incubation with a 1:200 dilution of mouse monoclonal anti-human VCAN (clone: 2B1; Seikagaku Corporation Ltd, Tokyo, Japan) for 25 minutes at room temperature (RT).

Immunolocalization of CD31 was performed on 56 paraffin-embedded tissue sections from patients with advanced stage serous ovarian cancers and 10 sections from normal ovaries. The same protocol as mentioned above was utilized. Sections were incubated with a 1:30 dilution of monoclonal mouse antihuman CD31 antibody (Fischer Scientific, Pittsburgh, PA) for 1 hour at RT.

VCAN protein expression was quantified using Image-Pro Plus 5.1.0.20 (Media Cybernetics, Bethesda, Maryland). The staining saturation was measured from three fixed-size areas in both the tumor epithelium and the tumor stroma. An average score was calculated for the epithelial and stromal components yielding 2 scores for each case.

The number of CD31-positive microvessels, or microvessel density (MVD), was determined by manually counting the number of positive cells per high power field (10X) by Image Pro

Plus 5.1.0.20. From each section, 3 areas were studied. Stromal and epithelial VCAN staining intensities were used to correlate with mean MVD.

Quantitative real-time PCR (Q-RT-PCR)

Q-RT-PCR was performed to evaluate VCAN mRNA expression in microdissected serous tumor samples. Expression was examined in both tumor epithelium and stroma of each sample (n = 65). VCAN expression was also examined in normal ovarian stroma (n=10). cDNA was generated from 15 nanograms of total RNA from the microdissected samples utilizing the WTovation RNA amplification system for RT-PCR (NuGen Technologies, Inc., San Carlos, CA). Q-RT-PCR was performed using the 7300 Real-time PCR System and TaqMan Gene Expression Assay probes (Applied Biosystems, Foster City, CA) for total VCAN(Hs01007940_m1) V0 (Hs 01007944_m1), V1 (.Hs01007937_m1), V2 (Hs01007943_m1), V3 (Hs01007941_m1), cyclophilin (NM_021130.3). The relative VCAN isoform level of each sample was normalized with endogenous cyclophilin A and calculated using $2^{-\Delta\Delta CT}$ by comparing the amount of expression to a HOSE sample [13].

Detection of VCAN by Western blotting

CHO K1 and CHO V1 culture medium was harvested from 1×10^6 cells/mL at 48 hours after plating. To inhibit proteolytic degradation of VCAN, one protease inhibitor tablet (Roche Applied Biosciences, Indianapolis, IN) was added per 10 mL of harvested medium. The harvested medium was concentrated 25-fold using Vivaspins 20 centrifuge tubes (Sartorius Stedim Biotech, Bohemia, NY) with a molecular weight cutoff of M_r 300,000 at 4°C for approximately 10-30 minutes at 6500 rpm. The concentrated samples were stored at -70°C.

To permit electrophoretic migration of VCAN (M_r ~900,000) through a 4-12 % polyacrylamide gel, the chondroitin sulfate side-chains were cleaved enzymatically from the core protein (M_r ~400,000) using chondroitinase ABC as previously described [14]. Western blot analysis was performed using a rabbit anti-V1 antibody provided by Prof. LeBaron (1:1000 in Tris-buffered saline/0.1% and 1% milk) for 2 hr. Visualization was achieved by an anti-rabbit IgG horseradish peroxidase-linked secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and the ECL Chemiluminescence System (Amersham Biosciences, Piscataway, NJ).

Western blot analysis was also performed on CHO V1 and K1 cell lysates to confirm V1 expression using β -actin for protein loading normalization. Cell lysates and conditioned media prepared from serous epithelial ovarian cancer cell lines mentioned above were also evaluated by the same approach.

Invasion Assay

The Cytoselect 24-well Invasion Assay was used as per manufacturer's instructions. (Cell Biolabs, Inc., San Diego, CA). After 48 hrs, 500 μ L of filtered media from CHO K1 and CHO V1 cells were collected and added to the lower well of the migration plate. OVCA3, OVCA420, OVCA429 (V1- cell lines), SKOV3 (V1+ cell line), and HUVEC cell lines were starved overnight. Three hundred microliters of cell suspension of each cell line ($0.5-1.0 \times 10^6$ cells/mL) were added to each insert. The plates were incubated for 24 hrs. Experiments were performed in triplicate.

Cell Proliferation Assay

After 48 hrs of incubation, filtered media from CHO K1 and CHO V1 cells was collected. Serous ovarian cancer lines from V1+ (SKOV3) and V1- expressing cells (OVCA3, OVCA420, OVCA429) and HUVEC were treated with CHO K1 and CHO V1 culture media

at various time points (0, 24, 48, and 72 hrs). Cell growth was determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. Experiments were performed in triplicate.

Statistical Analysis

The Kaplan-Meier method and log-rank statistics were used to estimate survival. The Mann-Whitney and Kruskal Wallis tests compared median of continuous variable between two or more independent samples respectively. The Spearman rank correlation assessed the relationship between two continuous variables. All statistical analyses were done with PASW 18 release 18.0.0 (SPSS, Inc., Chicago, IL). $P < 0.05$ was considered statistically significant.

RESULTS

Identification of differential VCAN expression on ovarian cancer stroma

Microarray analysis of microdissected advanced stage serous tumor epithelium ($n=16$), tumor stroma ($n=13$) and normal ovarian stroma ($n=10$) revealed multiple genes highly expressed in the tumor stroma, but not in the tumor epithelium or normal ovary (Fig S1). One of these genes was VCAN. The microarray data was validated by Q-RT-PCR of an independent set of microdissected tumor epithelium ($n=36$), tumor stroma ($n=13$), and normal ovarian stroma ($n=10$). There was a significantly higher expression of VCAN in the tumor stroma versus the tumor epithelium and the normal ovarian stroma ($P = 0.001$).

To further evaluate differential VCAN isoforms expression in ovarian cancer, Q-RT-PCR was performed on 8 microdissected advanced stage epithelial ovarian tissue samples to screen for the differential VCAN isoform expression. V1 was the most highly expressed isoform in these samples ($P = 0.008$) (Fig. 1A). Q-RT-PCR was then performed on 65 microdissected advanced stage serous tumor stroma and epithelium, 10 normal ovaries, and 1 HOSE sample. V1 mRNA was significantly higher in the tumor stroma versus the tumor epithelium ($P < 0.001$) and normal ovaries ($P < 0.001$) (Fig. 1B).

VCAN protein up-regulation and clinical correlations in advanced stage serous ovarian carcinoma

Immunolocalization of VCAN was performed on 111 serous ovarian tumors and 10 normal ovaries. The mean age of the patients was 58.5 years (range, 33-84). Expression of VCAN was significantly higher in the tumor stroma versus the tumor epithelium ($P < 0.01$). VCAN was expressed only by blood vessels noted in the normal ovary samples ($P < 0.01$).

For VCAN Kaplan Meier analysis, patients were divided into two groups (high versus low VCAN expression) using the 75th percentile for the VCAN expression analysis as the cut point. Patients with high VCAN expression of tumor stroma ($n=30$) had a median overall survival (OS) of 37.5 months [95 % confidence interval (95% CI), 25.7-49.3 months], whereas those with low VCAN expression ($n=72$) had a median OS of 79.8 months (95% CI, 61.7-91.7 months; $P = 0.001$; Fig. 2A). Patients with high VCAN expression of tumor stroma ($n=24$) had a median progression-free survival (PFS) of 9.5 months [95 % confidence interval (95% CI), 4.1-14.8 months], whereas those with low VCAN expression ($n=68$) had a median PFS of 37.6 months (95% CI, 19.8-55.4 months; $P = 0.008$; Fig. 2B).

In the subset of patients who were treated with platinum-based adjuvant chemotherapy, platinum resistance correlated with overexpression of VCAN in cancer-associated stroma ($P = 0.013$).

VCAN protein expression and vascularity

To evaluate vascularization, MVD was examined in 58 of the 111 serous ovarian tumors. The results showed that stromal VCAN expression was significantly correlated with MVD ($P < 0.01$, $r = 0.611$) (Fig. 3). This correlation was also shown through immunohistochemistry (Fig. 4). Immunolocalization of VCAN in a normal ovary and of a mouse IgG antibody instead of the mouse anti-VCAN in a high-grade serous ovarian cancer were used for negative controls (Fig. S2).

Western blot analysis of VCAN

Western immunoblotting was performed to evaluate V1 protein expression in serous ovarian cancer cell lines and to confirm V1 expression in the lysate and conditioned media of CHO V1 cells and lack of V1 expression in CHO K1 cells. The results showed that V1 expression was detected in CHO V1 media and not in CHO K1 media (Fig S3). Furthermore, V1 protein expression was detected in SKOV3 and SKOV3-i-luc lysates and conditioned media, but not in other serous cell lines (data not shown).

Effect of V1 conditioned media on serous ovarian cancer cell proliferation and invasion potential

To determine how exogenous V1 would affect ovarian cancer cell proliferation, two serous V1- ovarian cancer cell lines (OVCA3 and OVCA420), one V1+ serous ovarian cancer cell line (SKOV3), and an endothelial cell line (HUVEC) were treated with V1 conditioned media. Cellular proliferation was measured by the MTT assay. OVCA3, OVCA420, and HUVEC cells treated with the V1 media did not show significantly increased proliferation rate compared to that treated by the control medium-treated cells ($P = 0.468, 0.564, 0.710$ respectively). In contrast, SKOV3 cells treated with V1 media showed a significant increase in proliferation rate compared to cells treated with the control medium ($P = 0.038$) (Fig. 5).

To determine how exogenous V1 would affect ovarian cancer cell and endothelial cell invasion, three V1- serous ovarian cancer cell lines (OVCA3, OVCA420, OVCA429), one V1+ serous ovarian cancer cell line (SKOV3), and an endothelial cell line (HUVEC) were treated with V1 conditioned media. Cellular invasive potential was measured by the Cytoselect Invasion Assay. All five cell lines had increased invasion potential after being treated with V1 conditioned media ($P = 0.008$) (Fig. 6).

DISCUSSION

Using microdissected advanced stage serous tumor epithelium, tumor stroma and normal ovarian stroma; we identified a gene signature for the tumor stroma. One of the genes that showed significance is VCAN. VCAN is a large chondroitin sulfate proteoglycan belonging to the lexicon family. Four VCAN isoforms have been identified. The full-length isoform is V0, and smaller isoforms V1, V2, and V3 result from alternative splicing [3]. VCAN has been found in many malignancies including melanomas, prostate, and brain tumors [15]. Differential expression of VCAN isoforms has been shown in multiple human cancers. This differential expression is a component of the human melanoma cell differentiation process and well as in brain tumors [16,17]. In ovarian cancer, VCAN has been shown to be present in cancer cells and peritumoral stroma [18]. High stromal expression in epithelial ovarian cancers was associated with high FIGO stage, < 2 cm of residual tumor after surgical cytoreduction, serous histology, and reduced 5-year survival [18]. However, this study examined all stages and histologic subtypes and grades of epithelial ovarian cancer. Since the majority of patients presenting with epithelial ovarian cancer are advanced stage serous subtypes, we undertook this study to examine the role of VCAN in advanced stage serous

ovarian cancer by investigating its expression, function, and correlation with clinical outcomes.

We showed that VCAN protein was overexpressed in the tumor stroma of advanced stage serous ovarian tumors. This overexpression was associated with shortened PFS and OS. VCAN overexpression in these tumors was also associated with platinum resistance which has never been reported previously. Tumor stroma interactions inducing chemoresistance has been seen in pancreatic cancer, chronic and acute leukemias, and multiple myeloma [19-21]. *In vivo* studies of inhibitors to stromal factors leading to chemoresistance in leukemias have shown enhanced chemotherapy-induced apoptosis [21]. *In vivo* studies of pancreatic cancer revealed a mechanism of chemoresistance involving IL-1 β -triggered nitric oxide secretion by fibroblasts [19]. Further study of the VCAN's role in ovarian cancer platinum resistance is needed.

We also were able to show a correlation between VCAN overexpression and increased MVD and through functional studies V1's ability to increase invasiveness of endothelial cells. Studies have previously shown that VCAN has angiogenic properties [22,23]. Utilization of VCAN inhibitors to enhance the activity of anti-angiogenic biologics used in adjuvant treatment, such as bevacizumab, should be investigated in the future.

Examining the differential expression of VCAN isoforms in our microdissected serous ovarian tumors revealed that the V1 isoform is most highly expressed. Though other studies have reported overexpression of VCAN in ovarian cancer, V1 overexpression in advanced stage serous ovarian cancer is being reported here for the first time [18]. The predicted molecular weight (263kDa) and hyperglycosylation of the V1 isoform made a recombinant V1 protein difficult to produce. The V1 conditioned media from the CHO V1 cells was used as a surrogate. The functional studies have shown increased invasiveness in ovarian cancer cells treated with V1 protein and a trend toward increased cell proliferation in V1+ serous cell lines treated with V1 protein. Previous studies have shown that VCAN-treated cancer cells incorporate VCAN and hyaluronan (HA) into a pericellular matrix that promotes their motility and correlated with the ability to express HA receptor, CD44 [24]. Expression of CD44 is associated with clinically aggressive behavior in some human tumors. In ovarian cancer, CD44-expressing cells trigger cross-talk between different signaling pathways [25] possibly a mechanism for the peritoneal invasiveness of ovarian cancer. V1 in advanced stage ovarian tumors may bind to CD44 contributing to the extensive peritoneal spread of the disease. Other signaling and transcription factors that regulate VCAN expression include tyrosine kinase dependent pathways, the Wnt pathway, the phosphatidylinositol 3-kinase-protein kinase B pathway, and the sonic hedgehog pathway [26]. Clarification of the molecular and transcriptional partners for VCAN will hopefully define molecular targets for therapeutic interventions in ovarian cancer.

In summary, this study serves to begin to elucidate the expression and significance of VCAN, specifically the V1 form, in advanced stage serous ovarian cancer. Further study will be necessary to evaluate the potential of V1 inhibitors to act as an adjuvant biologic therapy to improve outcomes of traditional platinum/taxane-based therapy or to be a novel adjuvant therapy in recurrent platinum-resistant disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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A

B

Figure 1.

A. Quantitative RT-PCR analysis of VCAN mRNA isoform expression in advanced stage serous ovarian tumor samples. mRNA isolated from tumor epithelium (t) and tumor stroma (s) were analyzed separately. V1 was the most highly expressed isoform in these samples ($P=0.008$)

B. Q-RT-PCR analysis was performed on 65 microdissected advanced stage serous tumor stroma and epithelium, 10 normal ovaries, and 1 HOSE sample. V1 mRNA was significantly higher in the tumor stroma versus the tumor epithelium and versus normal ovaries ($P<0.001$)

Figure 2.

A. Kaplan Meier survival analysis. High VCAN expression in tumor stroma (median overall survival, 37.5 mos) versus low VCAN expression in tumor stroma (median overall survival, 79.8 mos). $P=0.001$. B. High VCAN expression in tumor stroma (median progression-free survival, 9.5 mos) versus low VCAN expression in tumor stroma (median progression-free survival, 37.5 mos). $P=0.008$. Cutoff for high expression of VCAN was 75th percentile.

Figure 3.
Scatter plot showing the correlation between tumor stromal VCAN expression and microvessel density ($P < 0.01$, $r = .611$)

Figure 4. Immunohistochemical staining of (A) CD31 and (B) VCAN in a case with low VCAN expression versus immunohistochemical staining of (C) CD31 and (D) VCAN in a case with high VCAN expression. Bar = 10 μ m.

Figure 5.

Effects of treating ovarian cancer cell lines with V1 on cell proliferation OVCA3 and OVCA420 (V1- cells lines) and HUVEC cells treated with the V1 media did not show significantly increased proliferation rate ($P= 0.468, 0.564, 0.710$ respectively). In contrast, SKOV3 cells (V1+ cell line) treated with V1 media showed a significant increase in proliferation rate . ($P=0.038$) (Fig. 5)

Figure 6.

Effects of treating ovarian cancer cell lines OVCA3, 429, and 420 (V1- cell lines), SKOV3 (V1+ cell line) and HUVEC cells with V1 on cell invasiveness. All five cell lines treated with V1 conditioned media showed a significant increase in invasive potential ($P = 0.008$).