Upregulation of CD9 in ovarian cancer is related to the induction of TNF-α gene expression and constitutive NF-κB activation

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Ovarian cancer is a gynecological cancer with a high death rate. We utilized global gene expression profiles of ovarian carcinomas obtained by complementary DNA (cDNA) microarray to identify ovarian cancer-specific proteins. CD9 was upregulated in ovarian carcinomas, and overexpression of the CD9 protein was detected in ovarian carcinomas by immunohistochemistry. CD9 was also overexpressed in several cancer cell lines, including ovarian cancer cells. In order to elucidate the biological significance of highly expressed CD9 in cancer cells, functional studies of CD9 were performed by ectopic expression, knockdown of CD9 using small interfering RNA (siRNA) and blockage of CD9 activity using the CD9-specific monoclonal antibody ALB6. Ectopic CD9 induced cell survival. In order to identify signaling pathways related to CD9, the gene expressions of CD9/SKOV3 cells were analyzed by cDNA microarray. Among the many upregulated genes, tumor necrosis factor (TNF)-α was induced in CD9/SKOV3 cells. The effect of overexpressed CD9 on the downstream signaling events of TNF-α was further investigated. In CD9/SKOV3 cells, the nuclear factor-kappaB (NF-κB)-signaling pathway was constitutively activated. Knockdown of CD9 by siRNA and blockage of CD9 activity by ALB6 in ovarian cancer cells demonstrated that constitutive activation of NF-κB is CD9 dependent and that CD9 is involved in anti-apoptosis. A CD9 functional study was performed in an ovarian cancer-xenograft mouse by injecting ALB6 into the peritoneum. ALB6 resulted in reduced tumor weight compared with that of control IgG1. Collectively, these results demonstrate that CD9 functions as an oncogene and represents a target for the development of cancer-specific therapeutics.

Introduction

Ovarian cancer is characterized by late diagnosis and a high level of recurrence, resulting in a high death rate (1,2). Most ovarian cancer is derived from the epithelial surface of the ovary; among the epithelial subtypes, papillary serous ovarian cancer is the most common and aggressive. At present, there are no ovarian cancer-specific early diagnostic biomarkers or targeted therapeutic agents. Paclitaxel and platinum-related drugs, such as cisplatin, are currently used to treat ovarian cancer. Because ovarian cancer patients experience frequent recurrence with a low 5 years survival rate, cancer-specific therapies are in high demand. Gene expression profiles in ovarian carcinoma tissues have been examined in an attempt to identify cancer-specific molecular markers (1–3).

CD9 is a cell surface glycoprotein that belongs to the tetraspanin family (4). The tetraspanin family has four transmembrane regions and two extracellular loops with which it interacts with many proteins, such as integrins, membrane-anchored growth factors and other tetraspan family proteins. These interactions facilitate functional protein complex formation, acting as a cell surface protein organizer (5). CD9 is expressed primarily in B cells and platelets as well as on the ovarian surface, where it plays a pivotal role in egg-sperm fusion (6). Recently, CD9 has been actively studied because of its up- or down-regulation in cancer tissue, with focuses on expression levels and cancer prognoses. Low CD9 expression is related to poor prognosis in colon (7), breast (8,9), lung (10,11) and pancreatic cancer patients (12). In contrast, head and neck squamous cell carcinoma (13), small-cell lung cancer (14) and gastric cancer (15) all express higher levels of CD9 in late stages. Some studies report that CD9 is downregulated in ovarian cancer and that lower expression of CD9 is related to poor prognosis (16,17), whereas others have found that CD9 is an ovarian cancer marker protein (3,18).

The function of CD9 has been studied in various cancers. Ectopic expression of CD9 in cancer cells expressing low levels of CD9 results in the inhibition of cell growth and motility, along with downregulation of the Wnt signal pathway that leads to the potential suppression of cell transformation and metastasis (4,7,11,19). At the same time, overexpression of CD9 in cancer has been linked to increased invasion and migration, tumorigenicity and chemoresistance (14,20). Furthermore, CD9 has recently been identified as a cancer stem cell marker in B-acute lymphoblastic leukemia (20,21), male germ cell cancer (22,23) and adipose-derived mesenchymal stem cells (24). Accordingly, CD9 may play a pivotal functional role in oncogenesis.

In this study, we report the overexpression of CD9 in ovarian carcinoma tissues and cancer cell lines. We demonstrate that enhanced CD9 is associated with anti-apoptosis, induced pro-inflammatory cytokine tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-8 expressions as well as constitutive activation of the nuclear factor-kappaB (NF-κB)-signaling pathway. The inhibition of CD9 with neutralizing monoclonal antibody reduced tumor weight in ovarian cancer-xenograft mice. We propose that CD9 offers a therapeutic target to treat solid tumors overexpressing CD9.

Materials and methods

Antibodies

The following antibodies were used for immunoblotting: anti-C9D9 (ALB6; Beckman Coulter), anti-Flag (M2, Sigma), anti-f-actin (Sigma), anti-α-tubulin (Santa Cruz Biotechnology), anti-GAPDH (Santa Cruz Biotechnology), anti-Lamin B (Santa Cruz Biotechnology) and anti-p65 (Santa Cruz Biotechnology). Mouse IgG1 (Sigma) was used as a control for ALB6. For immunofluorescence microscopy, Alexa Fluor 568 anti-rabbit (Molecular Probes) and Alexa Fluor 488 anti-rabbit (Molecular Probes) were used. For immunohistochemistry, anti-CD9 antibody was obtained from Chemicon (Millipore).

Cell lines

The human cell lines used in this study were HEK293 (embryonic kidney cell), U301 (renal cell carcinoma), NCI-H322m (non-small-lung cancer cell), MOLT-4 (T-cell leukemia cell), MCF-7 (breast cancer cell), HeLa (cervical cancer), colon cancer cell lines (COLO-205, HCT15, HCT116 and SW620) and ovarian cancer cells (MDAH-2774, 2774, SKOV3 and Ovcar3). Each cell was maintained in the media suggested by American Type Culture Collection.

Complementary DNA microarray

Ovarian carcinoma tissues were obtained from Samsung Medical Center with the approval of the Institutional Review Board and were snap-frozen in liquid nitrogen. The 30 ovarian tumors included 10 borderline tumors
(5 mucinous-type and 5 serous-type), 4 mucinous carcinomas and 16 serous carcinomas. Histological diagnosis was made by pathologists at the Samsung Medical Center. Normal ovarian tissues were obtained from ovarian cancer patients. The total RNA was isolated by Trizol reagent (Invitrogen) according to the manufacturer’s protocol. The global gene expression profile was analyzed by complementary DNA (cDNA) microarray using the 17K cDNA Chip (Genomic Tree, Korea). Total RNA from normal ovarian tissue was used as a control in pairs with the cancer tissues or after pooling of the total RNA.

Reverse transcription–polymerase chain reaction
Vec/SKOV3, CD9/SKOV3 and 2774 cells were treated with ALB6 or IgG1 for 6 h. Total RNA was isolated using Trizol, cDNA was synthesized using SuperScript III reverse transcriptase and reverse transcription–polymerase chain reaction (RT–PCR) was performed using EF-Taq polymerase (SolGent, Korea). The primers used for TNF-α (F: 5'-ACAAGCCTGTAGCCCATGTT-3' / R: 5'-AAAGTAGACCTGCCCAGACT-3'), IL-6 (F: 5'-TGTAGCCGCCCCACACAAGCC-3' / R: 5'-GAAGAGCCCTCAGGCTGGACTGC-3') and IL-8 (F: 5'-ATGACTTCCAAGCCTGCCCCTGCT-3' / R: 5'-CTCAGCCCTCTTCAAAACTTCTCC-3') were obtained from Bioneer (Korea). The polymerase chain reaction products were visualized under ultraviolet light and the band density was measured by Quantity One software (Bio-Rad).

Transfection of small interfering RNA
To knock down endogenous CD9 expression, 200 nM CD9-specific small interfering RNA (siRNA) (5'-GACGUACUCCGAACCUUCA-3'; Bioneer) was transfected with Lipofectamine 2000. Scrambled siRNA was used as a negative control. Thirty-six hours after transfection, cells were analyzed for knockdown efficiency by western blot under non-reducing condition.

NF-κB reporter assay
SKOV3 stable cells were plated on 24-well plates, transfected with plasmids encoding NF-κB-responsive firefly luciferase (NF-κB-RE-Luc) and renilla luciferase (pRL-SV40), which is an internal control for normalizing transfection efficiency using Lipofectamine 2000. Twenty-four hours after transfection, cells were washed with phosphate-buffered saline, serum-starved in Opti-MEM overnight and then assayed for luciferase activity using the dual-luciferase reporter assay kit (Promega).

To study the effect of CD9 knockdown on NF-κB activity, 2774, MCF-7 and HeLa cells were transfected with siRNA. Twenty-four hours after transfection with siRNA, cells were transfected with NF-κB-RE-Luc and pRL-SV40. Seven hours after transfection, cells were serum-starved overnight and then assayed for luciferase activity.

All assays were performed in triplicate and repeated three times; the data are presented as means ± SEMs. Data were analyzed using one-way analysis of variance followed by the Newman–Keuls multiple comparison test. Results were considered to be statistically significant at $P < 0.05$.

Ovarian cancer xenograft mouse model
To prepare the 2774 cells stably expressing green fluorescent protein (GFP), 1 x 10^5 cells in a 100 mm dish were transfected with pEGFP-C1 (Clontech). Twenty-four hours after transfection, the medium was changed to Dulbecco’s modified Eagle’s medium growth medium containing 500 μg/ml G418. Approximately 2–3 weeks later, G418-resistant colonies were chosen and GFP-expressing cells were determined under fluorescence microscopy.

GFP-2774 cells (5 x 10^6 cells per mouse) were inoculated into the peritoneal cavities of BALB/C nude mice. ALB6 (2 mg/kg) or IgG1 was injected into eight mice for each antibody every 3–4 days for the following 3 weeks. Mice were then killed, and tumors were imaged and weighed. Tumors were used for immunohistochemistry using anti-mouse secondary antibodies.
Upregulation of CD9 induces TNF-α expression

Among the many genes that displayed altered expression, TNF-α expression was distinctly increased in CD9/SKOV3 cells. To confirm the cDNA microarray data, RT–PCR of TNF-α was performed in CD9/SKOV3 and Vec/SKOV3 cells (Figure 3B). Two different CD9/SKOV3 stable clones demonstrated higher TNF-α expression than did the Vec/SKOV3 cells. Proinflammatory cytokine gene expression, such as IL-6 and IL-8, is upregulated by TNF-α (25), so we also evaluated IL-6 and IL-8 expression in CD9/SKOV3 and Vec/SKOV3 cells by RT–PCR (Figure 3B). Consistent with CD9-induced TNF-α expression, IL-6 and IL-8 were also predominantly expressed in CD9/SKOV3 cells. CD9-induced IL-6 expression also resulted in increased IL-6 secretion, as demonstrated by an IL-6-specific enzyme-linked immunosorbent assay (Figure 3C). The concentration of secreted IL-6 was ~1341 pg/ml in CD9/SKOV3 cells (clone #14) and 50 pg/ml in Vec/SKOV3 cells.

The biological effect of CD9-induced TNF-α expression was further explored by studying NF-κB activation. Upon activation of NF-κB by TNF-α, NF-κB subunits (p65 and p50) translocate into the nucleus to activate transcription of genes involved in cell proliferation or differentiation (26). Accordingly, we evaluated NF-κB activity by detecting the nuclear localization of the p65 subunit. Subcellular fractionation of CD9/SKOV3 and Vec/SKOV3 cells showed that p65 was mainly localized in the nucleus in CD9/SKOV3 cells (Figure 3D). An NF-κB-RE-Luc reporter assay also demonstrated that NF-κB activity was ~6.5-fold greater in CD9/SKOV3 compared with the level in Vec/SKOV3 (Figure 3E). NF-κB-related genes are listed in Supplementary Tables S1 and S2 (available at Carcinogenesis Online) among the genes whose expression levels changed by ~2-fold in CD9/SKOV3 cells.

**Results**

**CD9 is upregulated in ovarian carcinomas**

To discover novel tumor targets, total RNA was extracted from ovarian carcinoma tissues and analyzed by cDNA microarray (Figure 1A). CD9 was highly expressed in borderline and serous-type ovarian carcinomas, and the high expression levels were confirmed by real-time polymerase chain reaction (Figure 1B). CD9 expression in ovarian carcinomas was 5- to 10-fold greater than that in normal ovarian tissue after normalization with GAPDH expression. We then examined CD9 protein expression in ovarian cancer tissues by immunohistochemistry and found that the CD9 protein level was also elevated in papillary serous ovarian cancer (Figure 1C). Immunohistochemistry revealed overexpression of CD9 on the cell membrane. CD9 expression level was also measured in several cancer cell lines by RT–PCR and western blot analysis (Figure 2A and B). Expression of CD9 was higher in ovarian cancer cell lines, including ovarian cancer cells. Among the ovarian cancer cell lines tested, 2774 cells expressed high levels of CD9, whereas SKOV3 cells expressed low levels of CD9. Cellular localization analysis of CD9 in 2774 cells demonstrated that CD9 was localized to the cell membrane (Figure 2C). Collectively, these data indicate that CD9 can act as a tumour-specific antigen.

**CD9 induces cell growth and activates the NF-κB-signaling pathway**

In order to study the function of CD9, Flag-CD9 was stably transfected into SKOV3 cells, which had low CD9 expression. We selected several clones expressing Flag-CD9 and analyzed the CD9 expression level in two different stable cell lines (clones #14 and #16) compared with that in the vector-transfected cells (Figure 3A). The cell viability of Flag-CD9 stable cells was measured by MTT assay (Figure 3A). Exogenously expressed CD9 induced cell growth compared with that in the vector-transfected cells. To explore CD9-related-signaling events, we then studied the effect of CD9 on global gene expression by cDNA microarray analysis of a CD9 stable cell line.

**Knockdown of CD9 by siRNA leads to inhibition of NF-κB activity**

In order to establish whether the increased NF-κB activity was CD9-specific, we knocked down endogenous CD9 in various cancer cell lines by transfection of CD9-specific siRNA and measured the NF-κB-signaling pathway. CD9-specific siRNA was transfected into several cancer cell lines that highly expressed CD9, and the knockdown efficiency was determined by western blot analysis (Figure 4A). CD9-specific siRNA efficiently reduced the CD9 protein level. Ectopic CD9 improved cell viability, and we analyzed whether knockdown of CD9 led to cell death. After siRNA transfection, caspase-3 activity was measured to evaluate apoptosis (Figure 4B). Upon CD9 siRNA transfection into 2774, MCF-7 and HeLa cells, caspase-3 activity was augmented. The increased caspase-3 activity was diminished by a caspase-3-specific inhibitor. These results demonstrate that CD9 was anti-apoptotic in action.

To study the effect of downregulation of CD9 on the NF-κB-signaling pathway, an NF-κB-RE-Luc assay was performed in cancer cells (Figure 4C). Transfection of CD9-specific siRNA caused 40, 53 and 82% reductions in NF-κB-RE-Luc activity in 2774, MCF-7 and HeLa cells, respectively, compared with the levels in control-siRNA-transfected cells. Inhibition of NF-κB activity by CD9-specific siRNA was also determined by subcellular fractionation of cells. CD9-specific siRNA-transfected 2774 and MCF-7 cells were fractionated, and nuclear translocation of p65 was detected by western blot analysis. As shown in Figure 4D, nuclear translocation of p65 was inhibited upon CD9-specific siRNA transfection into 2774 and MCF-7 cells. These data indicated that CD9 is responsible for the induction of the NF-κB-related-signaling pathway.

**ALB6 inhibits the NF-κB-signaling pathway**

We further studied the function of CD9 by blocking CD9 activity using the neutralizing antibody, ALB6, against NF-κB activation. CD9/SKOV3 and Vec/SKOV3 cells were treated with ALB6, and NF-κB activity was measured by detecting p65 nuclear translocation. ALB6 blocked CD9-induced nuclear translocation of p65 in CD9/SKOV3 cells compared with the control IgG1 (Figure 5A). The inhibition of NF-κB activation by ALB6 was also confirmed by subcellular fractionation (Figure 5B). We then examined whether
ALB6 inhibited CD9-induced TNF-α, IL-6 and IL-8 expressions. The gene expression levels of TNF-α and IL-6 were detected by RT–PCR, revealing that ALB6 inhibited CD9-induced TNF-α and IL-6 expressions by ~50% after normalization with GAPDH expression (Figure 5C). IL-8 expression was also inhibited by ALB6 (Supplementary Figure S1 is available at Carcinogenesis Online). In order to detect the inhibitory effect of ALB6 on endogenous CD9, 2774 cells were treated with ALB6 and IgG1. ALB6 inhibited TNF-α, IL-6 and IL-8 expression by 20, 40 and 15%, respectively (Figure 5D). Overall, ALB6 inhibited the function of both endogenous and exogenous CD9.

ALB6 successfully targeted the tumor and that the reduction in tumor weight was due to the inhibition of CD9 by ALB6.

**Discussion**

In order to discover cancer-specific targets, we analyzed gene expression profiles in ovarian carcinoma tissues. Among the many genes that were differentially expressed in ovarian carcinoma tissues, we focused on cell surface proteins. Cell surface proteins offer potential cancer-specific targets for imaging materials and treatments, including therapeutic antibodies (27). Therapeutic antibodies against cancer cell surface proteins have been successfully used to treat various diseases (27). We identified at least eight membrane proteins (CD9, FOLR1, CLDN4, CLDN7, ERBB3, TACSTD1, CDH1 and SPINT2) with genes that were significantly upregulated in ovarian carcinomas. Most of these genes have been extensively studied in relation to ovarian cancer (28–32). Several studies have reported a correlation between CD9 expression levels and cancer prognosis. Previous studies on CD9 expression levels and cancer progression in ovarian cancer have produced contradictory results (2,18). We found that CD9 expression was high in ovarian carcinoma, especially in serous-type ovarian cancer. Therefore, we focused on CD9 to characterize its functional significance in cancer. In this study, we demonstrated that CD9 may function as an anti-apoptotic protein. In order to study the mechanism by which CD9 improves cell survival, we analyzed genes regulated by ectopic CD9. **TNF-α** was largely upregulated in CD9/SKOV3 cells. Increased TNF-α expression is often found in cancers, including ovarian cancer, and is related to cancer progression (33,34).
Upon ligation of TNF-α on TNF-α receptor 1 (TNF-R1), NF-κB is translocated into the nucleus, where it activates many genes involved in cell growth, differentiation and expression of pro-inflammatory cytokines, such as IL-6 and IL-8 (25). We examined the downstream signal of the TNF-α-TNF-R1 complex in cells overexpressing CD9. Overexpression of CD9 led to constitutive NF-κB activation. Knockdown of CD9 using siRNA in cancer cells expressing high levels of CD9 led to the inhibition of NF-κB activity. Taken together, these results demonstrate that CD9 is related to constitutive NF-κB activation. Constitutive NF-κB activation has been demonstrated in many cancers (35). However, the exact mechanism has not yet been elucidated. It is reported that cell death-inducing reagents target the NF-κB/TNF-α pathway to induce apoptosis of SKOV3, Ovcar-3 and 2774 ovarian cancer cells (36–39), demonstrating that the NF-κB/TNF-α-signaling cascade is critical for the survival of these ovarian cancer cells. Further study is needed to identify a detailed mechanism for CD9-induced NF-κB activation.

In the CD9/SKOV3 cells, IL-6 and IL-8 expressions were also predominantly higher compared with that in Vec/SKOV3. The enhanced TNF-α, IL-6 and IL-8 expressions were reduced by a CD9-neutralizing antibody, demonstrating that the upregulation of these cytokines are CD9 dependent. The expressions of IL-6 and IL-8 are high in ovarian cancer, especially in recurrent cases, and the resulting increased angiogenesis leads to the progression of cancer (35,40). Therefore, the overexpression of TNF-α, IL-6 and IL-8 induced by CD9 may be one possible mechanism for CD9-mediated tumorigenicity. These CD9-induced cytokine expressions seem to be cancer cell-specific phenomena because exogenously expressed CD9 in HEK293 cells did not result in increased these cytokine expressions (our unpublished results).

It is interesting that CD9 expression was high in borderline ovarian tumors (BOTs) in this study. Because they are associated with a favorable prognosis, low malignant potential and non-invasiveness, BOTs have received little attention. Recent evidence indicates that BOTs may recur and that recurrent BOTs could develop into malignant ovarian cancer and even cause death (41). The functional effect of CD9 on recurrence of BOTs and on progression to malignant ovarian cancer deserves further study. Overexpression of CD9 is also found in ascites-derived stromal cells from ovarian cancer patients, cells that promote angiogenesis, tumorigenicity and chemoresistance (42). Accordingly, CD9 might be involved in tumorigenicity.

Tetraspanin family proteins other than CD9 with expressions that were up- or downregulated in ovarian carcinomas are listed in Supplementary Table S3, available at Carcinogenesis online. Since tetraspanins are known to interact with many proteins (43,44), further studies of the functional significance of tetraspanin proteins in ovarian cancer would be interesting.

In conclusion, CD9 was overexpressed in ovarian carcinomas. Ectopic expression of CD9 induced TNF-α, IL-6 and IL-8 and constitutive NF-κB activation. CD9 may trigger tumorigenicity through overexpression of cytokines and NF-κB activation in certain cancers. Consequently, anticancer drug therapies targeting CD9, such as with siRNA and therapeutic antibodies, could be useful to inhibit the oncogenesis of ovarian tumors, to treat BOTs and to maximize the outcome of chemotherapy in ovarian cancer by reducing chemoresistance.
Supplementary material

Supplementary Table S1–S3 and Figure S1 can be found at http://carcin.oxfordjournals.org/

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Conflict of Interest Statement: None declared.

References


![Fig. 5. CD9 antibody inhibited the NF-κB-signaling pathway. (A) Vec/SKOV3 and CD9/SKOV3 cells were treated with ALB6 or IgG1 for 3 h. Cells were immunostained with anti-p65 antibody and anti-rabbit Alexa488. 4',6-Diamidino-2-phenylindole was used for nuclear staining. (B) Inhibition of NF-κB activity by ALB6 was determined by subcellular fractionation of CD9/SKOV3 cells after treatment with ALB6 or control IgG1. (C) Total RNA was isolated from SKOV3-stable cells treated with ALB6 or IgG1, and RT–PCR was performed using primers for TNF-α, IL-6 and GAPDH. The left panel shows RT–PCR data for TNF-α, IL-6 and GAPDH. The right panel demonstrates the band density of TNF-α and IL-6 after normalization with GAPDH. (D) RT–PCR for TNF-α, IL-6, IL-8 and GAPDH was performed using cDNA synthesized from ALB6- or IgG1-treated 2774 cells. The right panel demonstrates the band density of TNF-α, IL-6 and IL-8 after normalization with GAPDH.](https://example.com/fig5)

![Fig. 6. ALB6 reduced tumor weight. (A) IgG1 and ALB6 antibodies were injected into the GFP-2774 xenograft mice. Tumors were visualized on the dorsal and ventral sides and after dissection by fluorescence microscopy. (B) Tumors from each mouse were collected and weighed. Tumor weight is represented by a bar graph [*P < 0.01 and **P < 0.001, as compared with phosphate-buffered saline (PBS)-treated mice]. (C) Tumors taken from xenograft mice were stained with anti-mouse secondary antibody and hematoxylin and eosin.](https://example.com/fig6)


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