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Targeting ILK and β4 integrin abrogates the invasive potential of ovarian cancer

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ABSTRACT

Integrins and integrin-linked kinase (ILK) are essential to cancerous invasion because they mediate physical interactions with the extracellular matrix, and regulate oncogenic signaling pathways. The purpose of our study is to determine whether deletion of β1 and β4 integrin and ILK, alone or in combination, has antitumoral effects in ovarian cancer. Expression of β1 and β4 integrin and ILK was analyzed by immunohistochemistry in 196 ovarian cancer tissue samples. We assessed the effects of depleting these molecules with shRNAs in ovarian cancer cells by Western blot, conventional RT-PCR, cell proliferation, migration, invasion, and *in vitro* Rac1 activity assays, and *in vivo* xenograft formation assays. Overexpression of β4 integrin and ILK in human ovarian cancer specimens was found to correlate with tumor aggressiveness. Depletion of these targets efficiently suppresses ovarian cancer cell proliferation, migration, and invasion *in vitro* and xenograft tumor formation *in vivo*. We also demonstrated that single depletion of ILK or combination depletion of β4 integrin/ILK inhibits phosphorylation of downstream signaling targets, p-Ser 473 Akt and p-Thr202/Tyr204 Erk1/2, and activation of Rac1, as well as reduce expression of MMP-2 and MMP-9 and increase expression of caspase-3 *in vitro*. In conclusion, targeting β4 integrin combined with ILK can instigate the latent tumorigenic potential and abrogate the invasive potential in ovarian cancer.

1. Introduction

Integrin signaling is regulated by complex interactions with a number of cytosolic proteins, including integrin-linked kinase (ILK). ILK is a ubiquitously expressed protein serine/threonine kinase that was initially discovered through its interactions with the β1 and β3 integrin subunits [1,2]. Although ILK initially named as a kinase, ILK acts as a central component of ILK–PINCH–Parvin complex at ECM adhesions mediating interactions with a large number of proteins via multiple sites including its pseudoactive site [3–6].

Now many studies have reported that ILK plays a role as an adaptor and signaling protein in various aspects of the oncogenic process through direct and indirect mechanisms during tumor progression [7,8]. Recent reports showed that aberrant ILK mediated signaling, due to overexpression or constitutive activation of the protein, leads to pathological alterations that ultimately result in malignant progression in a range of cancers [9,10].

ILK expression is increased in ovarian epithelial cancer relative to benign tumors and normal ovarian epithelium, correlates with increased tumor grade, and is stimulated by soluble factors in peritoneal tumor fluid through the activation of the downstream protein kinase B/Akt pathway [11,12]. In addition, a recent study showed that ILK directly mediated actin cytoskeletal rearrangements and cell migration and invasion through the concerted actions of phosphoinositide 3-kinase (PI3K)/Akt/Rac1 [13].

Meanwhile, β1 integrin expressed on metastatic ovarian cancer cells affects adhesion to the mesothelium. It has been proposed that ovarian cancer metastasis is regulated by β1 integrin binding to the fibronectin secreted by mesothelial cells [14]. More recent studies also demonstrated that high levels of α4β1 and or αvβ3 integrins were closely correlated with increased peritoneal metastasis and tumor proliferation in ovarian cancer, respectively [15,16].

Furthermore, the expression of α6 and β4 integrin subunits in serous ovarian carcinoma correlates with expression of the basement membrane protein laminin. In most solid ovarian tumors, expression of laminin is patchy or absent in the putative basement membrane zone surrounding the nest of epithelial tumor cells. In addition, neither laminin or the α6 or β4 integrin subunits are present on the surface of ovarian carcinoma cells isolated from the ascites fluid of most patients, regardless of whether they are present in the patient's solid tumor. Thus, it is possible that ovarian carcinoma epithelial cells may be released from the basement membrane of the ovary due to their deficit of α6 and β4 integrin

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subunits [17]. However, the role of α6 or β4 integrin subunits in modulating the phenotypic behavior of ovarian carcinoma cells has not been thoroughly investigated and is poorly understood.

Accordingly, we hypothesized that overexpression of ILK and integrin β subunits in highly oncogenic cancer cells is related to ovarian cancer progression. We determined if targeting these molecules has antitumor effects for ovarian cancer. Furthermore, we addressed the question of whether there is a complementary and synergistic advantage when these molecules were targeted alone or in combination in highly oncogenic human ovarian cancer cells.

2. Materials and methods

2.1. Preparation of ovarian cancer patient samples for immunohistochemistry

We examined medical records and archival slides from the collection of ovarian serous adenocarcinoma of the Gynecologic Oncology Files of Yonsei University College of Medicine in Korea. One hundred ninety-six samples of ovarian serous carcinomas were isolated between 1990 and 2003 and used to create tissue microarrays with 2-mm pores in 3.8-cm × 2.2-cm × 0.5-cm frames. The detailed immunohistochemistry procedures and antibody information are provided in Supplementary materials and methods. Immunostaining was graded and scored as follows: 0 signifies no staining; 1+ signifies weak, diffuse staining; and 2+ signifies strong, diffuse staining.

2.2. Cell lines and cell culture conditions

The TOV-112D and OV-90 cell lines were obtained from the American Type Culture Collection and cultured in a 1:1 mixture of MCDB 105 medium (Invitrogen) and Medium 199 (Hyclone) containing 15% fetal bovine serum (FBS; Gibco BRL), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Gibco BRL). The SK-OV-3, MCF-7, and MDA-MB-231 cell lines were obtained from the Korean Cell Line Bank (Seoul, Korea) and cultured in RPMI-1640 medium (Gibco BRL) containing 10% FBS (Gibco BRL), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Gibco BRL).

2.3. shRNA-mediated mRNA depletion

We used the SureSilencing shRNA plasmid for human ITGB1 (KH00650G for the GFP), ITGB4 (KH00680G for the GFP), and ILK (KH00737G for the GFP), and a scrambled sequence negative control plasmid (SABiosciences). The shRNA target sequences are listed in Supplementary Table S1. The cells were seeded and transfected using the Attractene Transfection Reagent (QIAGEN) according to the manufacturer's protocol. At 24, 48, 72, and 96 h after transfection with the shRNA plasmids, the cells were harvested and total RNA was extracted using an RNeasy Protect Mini Kit (QIAGEN). The SuperScript III Reverse Transcriptase kit (Invitrogen) was used to synthesize cDNA. Polymerase chain reactions (PCRs) were performed with HotStarTaq DNA polymerase (QIAGEN). Primers, product sizes, and PCR conditions are listed in Supplementary Table S2.

2.4. Western blot analysis

Equal amounts of cell extract were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Invitrogen). Blots were blocked with 5% non-fat dry milk freshly dissolved in 1× phosphate buffered saline with Tween 20 (PBS-T), and incubated for 1 h at room temperature with primary antibodies (provided in Supplementary materials and methods). The blots were probed with an enzyme (horseradish peroxidase)-linked secondary antibody in 1× PBS-T (1:1500–100,000) for 1 h at room temperature. Finally, chemiluminescent detection reagents were used to visualize the results. Western blot signal was analyzed by an image analysis program (Multi Gauge V3.0, FUJIFILM).

2.5. Flow cytometric analysis

Antibodies were added at the appropriate dilutions and incubated for 20 min on ice in the dark. Antibody information is provided in Supplementary materials and methods. Samples were analyzed on a BD FACSAria™ cell sorter (BD Biosciences).

2.6. Rac1 activity assay

Rac1 activation assays were performed 96 h after shRNA transfection using a Rac1 G-LISA™ Activation Assay kit (Cytoskeleton, Inc.) according to the manufacturer's instructions. We measured the level of active, GTP-loaded Rac1 protein in cell lysates by absorbance at a wavelength of 490 nm using a VERSAmax microplate reader (Molecular Devices).

2.7. Cell proliferation and viability analysis

Cell proliferation was measured 96 h after shRNA transfection using a Cell Counting Kit-8 (Dojindo Laboratories) according to the manufacturer's instructions. We measured sample absorbance at a wavelength of 450 nm using a VERSAmax microplate reader (Molecular Devices).

2.8. Cell migration and invasion assay

Cell migration and invasion were analyzed using an Oris™ cell migration and invasion assay kit (Platypus Technologies), following the manufacturer's instructions. The detailed procedures are described in Supplementary materials and methods. The migrated and invaded cells were stained with Calcein AM and detected with a Victor™ X5 Multilabel Plate Reader (PerkinElmer Life and Analytical Sciences) using 485/528 nm excitation/emission filters.

2.9. Xenograft tumorigenicity assay

Nude mice were purchased from Central Lab Animal, Inc. (Seoul, Korea) and maintained in accordance with the institutional guidelines of Yonsei University College of Medicine. All animal studies were performed according to approved experimental protocols. Tumor cells (1×10^6 cells in 0.2 ml PBS) were injected subcutaneously in the dorsal flank of 6-week-old female nude mice. Tumors were measured every 4 days. Tumor volume was calculated with an index of the growth rate using the following equation: volume = (width + length)/2 × width × length × 0.5236. The mice were sacrificed 60 days after inoculation of the cells, and metastatic lesions on the lungs and livers were counted macroscopically.

2.10. Statistical analysis

Student's *t*-test was used, and results were considered statistically significant if the null hypothesis was rejected with a *P*-value <0.05.