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The insulin-like growth factor 1 pathway is a potential therapeutic target for low-grade serous ovarian carcinoma

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Abstract

Objective—To validate the overexpression of insulin-like growth factor 1 (IGF-1) and its receptor (IGF-1R) in low-grade serous ovarian carcinoma (SOC), and to investigate whether the IGF-1 pathway is a potential therapeutic target for low-grade SOC.

Methods—Gene expression profiling was performed on serous borderline ovarian tumors (SBOTs) and low-grade SOC, and overexpression of IGF-1 in low-grade SOC was validated by RT-PCR and immunohistochemistry. The effect of exogenous IGF-1 on cell proliferation was determined in cell lines by cell proliferation assays, cell migration assays, and Western blot. Signaling pathways downstream of IGF-1 and the effects of the AKT inhibitor MK-2206 were investigated by Western blot analysis and by generating IGF-1R short hairpin RNA stable knockdown cell lines. Low- and high-grade cell lines were treated with the dual IGF-1R- and insulin receptor-directed tyrosine kinase inhibitor OSI-906, and cellular proliferation was measured.

Results—mRNA analysis and immunostaining revealed significantly higher IGF-1 expression in low-grade SOC than in SBOTs or high-grade SOC. In response to exogenous treatment with IGF-1, low-grade cell lines exhibited more intense upregulation of phosphorylated AKT than did high-grade cell lines, an effect that was diminished with IGF-1R knockdown and MK-2206 treatment. Low-grade SOC cell lines were more sensitive to growth inhibition with OSI-906 than were high-grade cell lines.

Conclusions—IGF-1 is overexpressed in low-grade SOC compared with SBOTs and high-grade SOC. Additionally, low-grade SOC cell lines were more responsive to IGF-1 stimulation

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and IGF-1R inhibition than were high-grade lines. The IGF-1 pathway is therefore a potential therapeutic target in low-grade SOC.

Keywords

Ovarian cancer; Insulin-like growth factor; Insulin receptor

Introduction

It is generally accepted that low-grade and high-grade serous ovarian carcinomas (SOCs) evolve via molecularly and genetically distinct pathways [1-3]. Whereas high-grade SOC are thought to arise *de novo*, a majority of low-grade SOC tend to originate from molecular precursors, more specifically, serous borderline ovarian tumors (SBOTs) [4, 5]. This notion is supported by the fact that SBOTs frequently recur as low-grade SOC [6, 7]. Singer et al. [8] observed a gradual accumulation of genetic mutations in the progression from SBOTs to low-grade SOC, whereas high-grade SOC harbored several mutations even in early stages of tumorigenesis, developing rapidly from surface epithelium—suggesting a dualistic mechanism for the development of serous ovarian carcinomas. As a result of these and other similar observations, many institutions have adopted a binary grading system for ovarian carcinoma, which is highly reproducible [9, 10].

Clinically, high-grade and low-grade SOC behave differently as well. Women with advanced-stage low-grade SOC have higher 5-year survival rates than women with high-grade SOC [11, 12], an observation that reflects the indolent nature of low-grade SOC. Even though low-grade carcinomas portend improved overall survival, they are relatively chemoresistant and more difficult to treat upon recurrence [13, 14], and most patients with low-grade SOC eventually succumb to the disease. Despite these histologic and clinical differences, patients with low-grade and high-grade SOC are currently treated with the same standard protocol of surgery followed by platinum- and taxane-based chemotherapy.

New therapeutic strategies and novel molecular targets are needed to improve the outcome of this patient cohort. Recently, interest has emerged in the insulin-like growth factor (IGF) pathway as a potential therapeutic target. Downstream effectors of the IGF pathway, including phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) and RAF/mitogen-activated protein (MAP) kinase, have well-established roles as mitogens in carcinogenesis [15, 16]. Several clinical trials are also under way exploring the efficacy of IGF-1 receptor (IGF-1R) inhibitors in various malignancies. OSI-906 (OSI Pharmaceuticals, Melville, NY) is one such inhibitor, which dually targets the tyrosine kinase domain of IGF-1R and the insulin receptor (IR).

By gene expression profiling, we found that IGF-1 is highly expressed in low-grade SOC in comparison to SBOTs. We hypothesize that the IGF-1 pathway is required for the pathogenesis of low-grade SOC and is a potential therapeutic target for low-grade SOC. In this study, we sought to validate the over-expression of IGF-1 in low-grade SOC, and to demonstrate that IGF-1 pathway as a potential therapeutic target for low-grade SOC.

Materials and Methods

Tissue samples

The Department of Pathology at The University of Texas MD Anderson Cancer Center provided archived formalin-fixed paraffin-embedded tissue samples from patients with SBOT, advanced-stage low-grade SOC, and advanced stage high-grade SOC. All cases were reviewed and classified as low-grade or high-grade by gynecologic pathologists (A.M. and

M.T.D.) using histologic criteria described previously [17, 18]. Frozen tissues were obtained from the Multidisciplinary Gynecologic Cancer Translational Research Tissue Bank at MD Anderson Cancer Center. All specimens had been collected, archived, and handled under protocols approved by the Institutional Review Board.

Cell lines and materials

We used one SBOT cell line (ML46), two low-grade SOC cell lines (HOC-7 and MPSC1) [19, 20], and four high-grade SOC cell lines (SKOV3, OVCA420, HeyA8, and 2774). ML46 and HOC-7 were a gift from Dr. Louis Dubeau at the University of Southern California; MPSC1 was a gift from Dr. Ie-Ming Shih at Johns Hopkins University. All other cell lines were authenticated by DNA techniques at MD Anderson. Cell line HOC-7 contains a KRAS mutation; cell line MPSC1 contains a BRAF mutation. AKT inhibitor MK-2206 was purchased from Selleck (Houston, TX) and tyrosine kinase inhibitor OSI-906 was purchased from ChemieTek (Indianapolis, IN). All cell lines were cultured in Cellgro minimum essential medium (Mediatech, Manassas, VA) with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were grown in a humidified incubator at 37°C with 5% CO₂.

Gene expression profiling

Total RNA were extracted from microdissected tumor cells from SBOTs (n = 8) and low-grade SOC (n = 13). Gene expression profiles were generated using GeneChip Human Genome U133 Plus 2.0 array (Affymetrix, Santa Clara, CA). The Two-Cycle cDNA synthesis kit was used for two rounds of amplification to generate sufficient levels of cRNA for microarray analysis using 25 ng total RNA. For the first round of synthesis of double-stranded cDNA, RNA was reverse-transcribed according to the manufacturer's instructions and then amplified with a MEGAscript T7 kit (Ambion Inc, Austin, TX). We performed clean up of the cRNA using a GeneChip Sample Cleanup Module In Vitro Transcription (IVT) column, followed by a second round of double-stranded cDNA amplification with the IVT labeling kit. A 15.0 µg aliquot of labeled product was fragmented by heat- and ion-mediated hydrolysis at 94°C for 35 min in 24 µL of H₂O and 6 µL of 5× fragmentation buffer. The fragmented cRNA was hybridized for 16 h at 45°C to a U133 Plus 2.0 oligonucleotide array in a GeneChip Hybridization Oven 640. A Fluidics Station 450 was used to wash and stain the arrays with phycoerythrin-conjugated streptavidin (Molecular Probes, Eugene, OR), and the arrays were then scanned using a confocal laser GeneChip Scanner 3000 and GeneChip operating software.

Quantitative real-time reverse transcriptase polymerase chain reaction

Overexpression of IGF-1 in low-grade SOC was validated by quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR). Total RNA extraction (RNeasy Mini kit, Qiagen, Germantown, MD) and cDNA synthesis (High Capacity cDNA Archive kit, Applied Biosystems, Carlsbad, CA) were performed according to the manufacturer's instructions. Laser microdissection was carried out on 42 samples: 9 SBOT, 18 low-grade SOC, and 15 high-grade SOC. Total RNA was extracted, and qRT-PCR was performed twice in triplicate using TaqMan primer sets for IGF-1 and for the housekeeping gene *cyclophilin* (Applied Biosystems), utilizing the Applied Biosystems 7300 system for all reactions. The fold change was calculated with the $\Delta\Delta C_t$ method described by Wong et al [21].

Immunohistochemical analysis

Paraffin sections 4 µm in thickness were cut from formalin-fixed paraffin-embedded specimens and mounted on glass slides. Slides were deparaffinized and rehydrated in

ethanol. Antigen retrieval was carried out with citrate buffer in a decloaking chamber (Biocare Medical, Concord, CA) heated to 120°C for 15 min. Background Sniper blocking reagent (Biocare Medical) was applied for 20 min, and Tris-buffered saline-rinsed sections were then incubated with antibodies. Rabbit monoclonal antibodies to IGF-1 (Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit monoclonal antibodies to IGF-1R (Ventana Medical Systems, Inc., Tuscon, AZ) were incubated with slides overnight at 4°C. Slides were then incubated with a two-step Mach 3 rabbit immunoglobulin G alkaline phosphatase kit (Biocare Medical). Slides were then counterstained with Hematoxylin QS (Vector Laboratories, Burlingame, CA) and fixed with Permount mounting medium (Biomedica Corp, Foster City, CA). Slides were evaluated by light microscopy at 100× and 200× magnifications, with photographs taken at 200× to document staining intensities.

Generation of stable IGF-1R knockdown cell lines

Lentiviral particles carrying IGF-1R targeted short hairpin RNA (shRNA) were purchased from Sigma Aldrich (St. Louis, MO). The sequences used to silence IGF-1R are listed in Supplemental Table 1. Infection of HOC-7 cells with five shRNAs and one nontarget shRNA was performed according to manufacturer's instructions.

Western blot analysis

Total protein was extracted from plated cells with radioimmunoprecipitation assay buffer to obtain protein lysates. HOC-7, MPSC1, 2774, and SKOV3 cells were treated with IGF-1 at a concentration of 100 ng/ml for 1 h, and then lysates were extracted from control (untreated) and treated cells. Lysates were also created from HOC-7, nontarget shRNA, and IGF-1R targeted shRNA stable cell lines with and without the addition of 100 ng/ml of IGF-1. HOC-7 cells were plated with untreated HOC-7 controls, and after 24 h, both were treated with various concentrations (1 μM, 2 μM, 4 μM, and 6 μM) of the AKT inhibitor MK-2206 and then treated with 100 ng/ml of IGF-1, and lysates were collected after 5 h. Protein lysates (10 μg) were electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel, electroblotted onto a polyvinylidene difluoride membrane, and blocked in 5% nonfat milk in phosphate-buffered saline with 0.05% Tween 20 (PBS-T) for 1 h. Western blots were incubated overnight at 4°C with the primary antibody to IGF-1R, phosphophorylated AKT (pAKT), AKT (1:1000, Cell Signaling, Danvers, MA) and β-actin (1:3000, Sigma, St. Louis, MO) in 1× PBS-T with 5% bovine serum albumin. Next, the blots were incubated with a horseradish peroxidase-conjugated secondary antibody (1:1000; Amersham-GE Healthcare) diluted in PBS-T with 5% nonfat milk for 1 h at room temperature. Amersham ECL Plus detection reagent (GE Healthcare, Waukesha, WI) was used to visualize protein bands.

Cell proliferation and migration assays

ML46, HOC-7, MPSC1, SKOV3, and 2774 cells, were plated at a density of 1×10^3 cells per well in 96-well plates for 24 h and exposed to various concentrations of IGF-1 (10 ng/ml, 25 ng/ml, 50 ng/ml, and 100 ng/ml). After incubation for 24 h, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to each well, and the number of viable cells was determined by measuring the absorbance at 570 nm using a microplate reader (BMG Labtech, Chicago, IL). Similarly, high-grade SKOV3, OVCA420, and HEYA8 cells and low-grade MPSC1 and HOC-7 cells were plated at a density of 1×10^3 cells per well in 96-well plates for 24 h and exposed to various concentrations of OSI-906 (1 μM, 3 μM, 5 μM, 7 μM, 9 μM, and 10 μM). After incubation for 72 h, WST-1 (Roche, Pleasanton, CA) cell proliferation reagent was added to each well, and the number of viable cells was determined by measuring the absorbance at 450 nm using a microplate reader (BMG Labtech, Chicago, IL).

The low-grade MPSC1, shRNA control, and IGF-1R shRNA knockdown MPSC1 cells 39675 and 121301 were plated at a density of 9×10^5 on 10-cm plates with a rubber stopper in the center of the plate and were grown until 90% confluent. The stopper was then removed, and cells were treated with 100 ng/ml of IGF-1. Cell migration was documented with photography at 0, 6, 24, and 30 h.

Statistical analysis

Analyses were carried out using statistical package SPSS 15.0 for Windows (SPSS Inc, Chicago, IL). A nonparametric Mann-Whitney *U* test was used to assess the statistical significance of the difference in IGF-1 mRNA expression between normal human ovarian surface epithelial cells and microdissected ovarian cancer tissues. A *p* value < 0.05 was considered statistically significant. Immunohistochemical staining was quantified according to H-score as previously described [22]. Median H-scores for each histological subtype were calculated, and were categorized as strong (135-300), moderate (26-124), or weak (0-25) staining intensity.

Results

IGF-1 is upregulated in low-grade SOC compared to SBOTs

To identify genes upregulated in low-grade SOC, differential gene expression analysis was performed as described previously [23]. The analysis identified several genes that were differentially expressed between low-grade SOC and SBOT, the top 20 of which are listed in Supplemental Figure 1. *IGF-1* was among the most overexpressed genes. This overexpression of *IGF-1* in low-grade SOC samples was validated by RT-PCR (Figure 1). Comparisons of *IGF-1* mRNA expression between SBOT, low-grade SOC, and high-grade SOC samples revealed significantly higher IGF-1 expression in low-grade SOC than in SBOTs (*p* < 0.0005) and high-grade SOC (*p* = 0.033).

IGF-1 and IGF-1R are overexpressed in low-grade serous ovarian carcinoma

In order to confirm IGF-1 and IGF-1R protein overexpression in low-grade SOC, immunostaining with IGF-1 and pAKT was performed. IGF-1R expression was evaluated in low-grade SOC (43 slides), as well as other histologic subtypes including clear cell (22 slides), mucinous (52 slides), and endometrioid (17 slides) ovarian carcinomas. Immunostaining of low-grade tumors with IGF-1 and pAKT revealed robust staining in the low-grade tumors, with less intense staining in the high-grade SOC slides and very weak staining in the SBOT slides (Figure 2). Low-grade SOC exhibited the strongest IGF-1R expression with a median H-score of 250, while endometrioid type ovarian carcinomas also exhibited strong staining (median H-score 150). Clear cell carcinomas exhibited less intense staining (median H-score 25), while IGF-1R expression was virtually absent in the mucinous carcinomas (median H-score 0), (Supplemental Figure 2).

IGF-1 activates the pAKT pathway in low-grade SOC and is inhibited by the AKT inhibitor MK-2206 and IGF-1R knockdown

In order to confirm that IGF-1 activates the pAKT pathway in ovarian cancer, we performed Western blot analysis of pAKT activation in ovarian cancer cell lines treated with exogenous IGF-1. Both of the high-grade cell lines tested (SKOV3 and 2774) expressed endogenous pAKT (Figure 3), whereas only one low-grade cell line (MPSC1) exhibited endogenous pAKT activity. In response to exogenous treatment with IGF-1, both of the low-grade cell lines (MPSC1 and HOC-7) exhibited intense upregulation of pAKT, whereas the high-grade cell lines exhibited a less drastic response (Figure 3A). Furthermore, activation of pAKT by IGF-1 in the low-grade cell line HOC-7 was blocked by the AKT inhibitor MK-2206

(Figure 3B). In the presence of IGF-1R shRNA knockdown, IGF-1 no longer activated pAKT (Figure 4), and the extent of IGF-1R knockdown correlated with pAKT expression. This confirmed successful knockdown of IGF-1R and activation of pAKT via IGF-1 and IGF-1R in HOC-7 low-grade cell lines.

Low-grade cell lines are more sensitive to stimulation with IGF-1

In cellular proliferation assays of ovarian cancer cell lines treated with exogenous IGF-1, cellular growth was most drastically increased in the low-grade cell lines MPSC1 and HOC-7 but was increased overall in all cell lines, including the high-grade cell lines 2774 and SKOV3 and the SBOT cell line ML46 (Figure 5). In cell migration assays, cellular migration was increased in the low-grade cell line MPSC1 versus controls when treated with exogenous IGF-1 (Supplemental Figure 3) and to a lesser extent in the presence of IGF-1R knockdown. Similar results were observed in a cell migration assay in HOC-7 cells (data not shown).

Low-grade cell lines are more sensitive to IGF-1R directed inhibition

When cells were treated with the tyrosine kinase dual IR and IGF-1R inhibitor OSI-906, cellular growth inhibition was observed in both low- and high-grade cell lines, but inhibition was most drastic in the low-grade cell lines (Figure 6).

Discussion

We have shown that IGF-1 is overexpressed in low-grade SOC cell lines at both the mRNA and protein level. Additionally, low-grade SOC cell lines are more responsive to IGF-1 stimulation than high-grade SOC cell lines. This is effected through IGF-1R and AKT activation and is blocked through IGF-1R knockdown and/or AKT inhibition. Correspondingly, low-grade SOC cell lines are also more sensitive to dual IGF-1R- and IR-directed inhibition with OSI-906. As predicted, IGF-1R protein expression via immunohistochemistry was highest in low-grade SOC cell lines. Interestingly, the endometrioid type ovarian carcinomas also exhibited robust staining. This subgroup may therefore also benefit from IGF-1R targeted therapy, and these findings merit further investigation.

Previous studies have validated the presence of the IGF-1 pathway in ovarian carcinomas; Yee et al were the first to describe IGF-1 mRNA and IGF-1R expression in ovarian cancer cell lines [24]. Others have shown that the IGF-1 pathway is upregulated in microarray analyses of ovarian carcinoma tissues and that this upregulation is inversely correlated with survival [25]. Whereas these studies have primarily examined samples with high-grade histologies, we have demonstrated that IGF-1 and IGF-1R are particularly overexpressed in samples with low-grade histologies, which has significant implications for treatment. Clinically, our novel discovery that the IGF pathway is upregulated in low-grade SOC cell lines provides a potential opportunity for targeted therapy for a disease that is difficult to cure upon recurrence. The concept that targeting the IGF pathway may be important in ovarian cancer is supported by demonstrations by others that IGF-2 plays a role in paclitaxel resistance and that IGF-2 expression predicts a poorer prognosis in SOC [26, 27]. The search for potential therapies targeting the IGF pathway has received particular attention in the study of sarcomas, colorectal and lung cancers, and other malignancies [28-30]. The utility of IGF-1R targeted therapy in ovarian cancer remains largely unexplored; the only available clinical data have been derived from phase I trials. While limited, the data are promising: Karp et al noted one complete response in an ovarian carcinoma patient in a phase I trial combining carboplatin and paclitaxel with the IGF-1R-directed monoclonal antibody figitumumab [31]. Three clinical trials investigating the efficacy of IGF-1R-directed therapy in ovarian carcinoma are under way. A phase II trial is currently

investigating the fully human anti-IGF-1R monoclonal antibody AMG 479 in combination with standard chemotherapy after optimal surgical cytoreduction (NCT00718523), and another phase II trial is examining AMG 479 in recurrent platinum-sensitive ovarian cancer (NCT00719212). NCT00889382 is currently evaluating OSI-906 in combination with weekly paclitaxel in patients with recurrent epithelial ovarian cancer in a phase I/II study.

The current clinical trials available to low-grade patients will likely recruit a majority of patients with the most prevalent histology, which is high-grade SOC. As low-grade and high-grade SOC are distinct entities, more trials aimed at low-grade histology are needed. The only current clinical trial specifically targeting low-grade SOC is investigating AZD6244, a MAP kinase kinase (MEK) inhibitor, in recurrent low-grade or peritoneal carcinoma (NCT00551070). Unfortunately, that trial is not currently recruiting patients. However, our data argue that targeting the IGF-1 pathway in low-grade SOC may yield more clinical benefit, and further investigation into IGF-1R-targeted therapy in low-grade serous ovarian carcinomas is therefore warranted. Unfortunately, the low-grade cell line HOC-7 is nontumorigenic in nude and severe combined immunodeficient (SCID) mice and we were unable to generate an orthotopic mouse model for testing these drugs. However, a genetic mouse model that will form low-grade serous ovarian carcinomas has recently been developed [32], and will be useful for future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Research Highlights

- Insulin-like growth factor 1 is overexpressed in low-grade serous ovarian carcinomas compared to high-grade serous ovarian carcinomas.
- Low-grade serous carcinomas are more sensitive to insulin-like growth factor 1 stimulation and insulin-like growth factor 1 receptor inhibition.
- Therefore, the insulin-like growth factor 1 pathway represents a novel potential therapeutic target for low-grade serous ovarian carcinoma.

Figure 1.

Box plot of IGF-1 mRNA expression in 9 serous borderline tumors and 18 low-grade and 15 high-grade SOC samples compared to human ovarian surface epithelium (HOSE). The box is bounded by the 25th and 75th percentiles, with the median expression level depicted by the line in the box. Outlying values are drawn individually. Expression of IGF-1 was significantly higher in low-grade SOC than in SBOT ($p < 0.0005$) or high-grade SOC ($p = 0.033$).

Figure 2. A-C. Representative examples of IGF-1 immunohistochemical staining of individual paraffin sections from SBOTs (2A), low-grade SOCs (2B), and high-grade SOCs (2C). D. Representative example of pAKT immunohistochemical staining of individual paraffin sections from low-grade SOCs (magnification: 200 $\times$).

Figure 3.

A. Western blot analysis of pAKT activation in ovarian cancer cell lines treated with exogenous IGF-1. B. Activation of pAKT by IGF-1 in low-grade ovarian cancer cell lines was blocked by the AKT inhibitor MK-2206 in a dose-dependent manner.

Figure 4.

IGF-1 no longer activated pAKT after shRNA IGF-1R knockdown. We performed five independent shRNA knockdowns and a nontarget shRNA control knockdown using the low-grade SOC cell line HOC-7. pAKT expression correlated with the extent of IGF-1R knockdown.

Figure 5. Cell proliferation assays in two low-grade SOC cell lines (HOC-7, MPSC1), two high-grade SOC cell lines (SKOV3, 2774), and one SBOT (ML46) cell line exposed to exogenous IGF-1 versus controls. Proliferation was higher in treated cells than in control cells for all cell lines, but the difference between control and treatment was most pronounced in the low-grade SOC cell lines.

Figure 6.

Cell proliferation assays in two low-grade (HOC-7, MPSC1) and three high-grade (SKOV3, HEYA8, OVCA429) SOC cell lines after treatment with various concentrations of OSI-906 expressed as a percentage of the proliferation in the control cells. Inhibition was most profound in the low-grade SOC cell lines.