Id1-Induced IGF-II and Its Autocrine/Endocrine Promotion of Esophageal Cancer Progression and Chemoresistance—Implications for IGF-II and IGF-IR–Targeted Therapy

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Abstract

Purpose: To investigate the autocrine/endocrine role of Id1-induced insulin-like growth factor-II (IGF-II) in esophageal cancer, and evaluate the potential of IGF-II- and IGF-type I receptor (IGF-IR)-targeted therapies.

Experimental Design: Antibody array-based screening was used to identify differentially secreted growth factors from Id1-overexpressing esophageal cancer cells. In vitro and in vivo assays were performed to confirm the induction of IGF-II by Id1, and to study the autocrine and endocrine effects of IGF-II in promoting esophageal cancer progression. Human esophageal cancer tissue microarray was analyzed for overexpression of IGF-II and its correlation with that of Id1 and phosphorylated AKT (p-AKT). The efficacy of intratumorally injected IGF-II antibody and intraperitoneally injected cixutumumab (fully human monoclonal IGF-IR antibody) was evaluated using in vivo tumor xenograft and experimental metastasis models.

Results: Id1 overexpression induced IGF-II secretion, which promoted cancer cell proliferation, survival, and invasion by activating AKT in an autocrine manner. Overexpression of IGF-II was found in 21 of 35 (60%) esophageal cancer tissues and was associated with upregulation of Id1 and p-AKT. IGF-II secreted by Id1-overexpressing esophageal cancer xenograft could instigate the growth of distant esophageal tumors, as well as promote metastasis of circulating cancer cells. Targeting IGF-II and IGF-IR had significant suppressive effects on tumor growth and metastasis in mice. Cixutumumab treatment enhanced the chemosensitivity of tumor xenografts to fluorouracil and cisplatin.

Conclusions: The Id1–IGF-II–IGF-IR–AKT signaling cascade plays an important role in esophageal cancer progression. Blockade of IGF-II/IGF-IR signaling has therapeutic potential in the management of esophageal cancer.

Introduction

Tumor-secreted growth factors can affect tumor micro-environment (1, 2), as well as stimulate the cancer cells themselves to proliferate and develop a more malignant phenotype in an autocrine manner (3–6). A variety of growth factors and cytokines exert their effects on cancer cells by activating the membrane-associated phosphoinositide 3-kinase (PI3K)/AKT signaling pathway, which regulates a wide spectrum of cellular functions, including proliferation, survival, angiogenesis, invasion, and metastasis. We previously reported that the PI3K/AKT signaling pathway can be induced by ectopic expression of Id1 (inhibitor of differentiation or DNA binding), leading to increased proliferation and survival of esophageal cancer cells (7). The Id1 protein is a helix-loop-helix protein (8) found to be overexpressed in many types of human cancer, including esophageal squamous cell carcinoma (ESCC; ref. 9). Our tissue microarray (TMA) study established that dysregulated Id1 expression is associated with tumor invasion and metastasis in ESCC (10).

The mechanism by which Id1 stimulates the PI3K/AKT pathway is not clear. In this study, using growth factor antibody array, we provided the first evidence that the PI3K/AKT signaling pathway can be induced by ectopic expression of Id1 (inhibitor of differentiation or DNA binding), leading to increased proliferation and survival of esophageal cancer cells (7). The Id1 protein is a helix-loop-helix protein (8) found to be overexpressed in many types of human cancer, including esophageal squamous cell carcinoma (ESCC; ref. 9). Our tissue microarray (TMA) study established that dysregulated Id1 expression is associated with tumor invasion and metastasis in ESCC (10).

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Translational Relevance

Esophageal cancer ranks as the sixth most frequent cause of cancer death in the world. There is an urgent need for a better understanding of the underlying mechanisms to develop more effective therapeutic strategies for this highly lethal disease. This study uncovers an autocrine/endocrine link between Id1 and the PI3K/AKT pathway involving IGF-II and IGF-IR, which promotes esophageal cancer progression and metastasis. Cixutumumab is currently investigated in clinical trials for the treatment of non–small cell lung cancer and hepatocellular carcinoma. Here, we show that systemic treatment with cixutumumab can significantly inhibit esophageal cancer growth and metastasis, as well as sensitize tumor xenografts to fluorouracil and cisplatin treatment in vivo. The present study therefore provides important preclinical data supporting the clinical application of cixutumumab systemic therapy both as a single agent and in combination with cytotoxic chemotherapeutic drugs.

The IGF system is an attractive target for cancer therapy (11, 12). Elevated expressions of insulin-like growth factor type I receptor (IGF-IR), and its ligands IGF-I and IGF-II, had been reported in human cancers, including ESCC, and were found to be correlated with advanced tumor stage and metastasis (13, 14), but treatment efficacy of IGF-IR blockade in ESCC remains to be evaluated. The feasibility of targeting the IGF-II-IGF-IR axis as potential treatment options for ESCC was investigated in this study in vitro, as well as in vivo using tumorigenesis and cancer metastasis mouse models. Because treatment failure and cancer recurrence may be attributed to chemoresistance during conventional chemotherapy, we also examined whether IGF-IR blockade can enhance the chemosensitivity of ESCC cells in animal models.

Materials and Methods

Cell lines and transfection

Human ESCC cell lines HKESC-3 (15), KYSE150, KYSE270, KYSE410 (16), and T.Tn (17) were used in this study. The Id1-overexpressing cell lines HKESC-3-Id1, KYSE150-Id1, and T.Tn-Id1 and their corresponding control cell lines HKESC-3-CON, KYSE150-CON, and T.Tn-CON were generated previously (18, 19). Details on reagents and plasmids used in gene overexpression and knockdown, and in generating KYSE150-Luc cell line, are given in Supplementary Materials and Methods.

Growth factor antibody array

Conditioned culture media from HKESC-3-Id1 and HKESC-3-CON cells were analyzed using a human growth factor antibody array that detects 41 growth factors (Catalog No. AAH-GF-1, RayBiotech) according to the manufacturer’s instructions. The immunosignals were detected with the ECL Plus system (Amersham) and exposed to BioMax Light Film (Kodak).

In vitro ELISA, quantitative real-time PCR, Western blot analysis, MTT, TUNEL, cell migration, invasion, and soft agar assays

The concentration of human IGF-II in the culture medium and mouse serum was measured using a Non-Extraction IGF-II ELISA Kit (Diagnostic Systems Laboratories) according to the manufacturer’s instruction. Anti-IGF-II (R&D Systems) was used in immunoneutralization experiments. Details of assays were described in our previous studies (19, 20). Western blot bands were quantified using ImageJ software. See Supplementary Materials and Methods for details of antibodies and real-time PCR.

In vivo tumorigenesis and metastasis experiments

Animal experiments were conducted and subcutaneous tumor volume and metastatic tumor nodules in the lungs of mice were quantified as previously described (19, 20). Details are provided in Supplementary Materials and Methods.

Immunohistochemistry

A TMA containing 35 cases of human ESCC in duplicated cores (Catalog no. ES802, Biomax), sections of tumor xenografts and mouse lung tissues were processed for immunohistochemistry and TUNEL (terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling) assay. Staining intensity (for Id1, IGF-II, and phosphorylated AKT; p-AKT), Ki-67 proliferative index, TUNEL-positive apoptotic index, and CD31-positive microvessel density (MVD) were assessed as previously described (19, 20). See Supplementary Materials and Methods for details.

Statistical analysis

The data were expressed as the mean ± SD and compared using ANOVA. Correlations of IGF-II, Id1 and p-AKT in the TMA were assessed using Spearman rank correlation coefficient. P values < 0.05 were deemed significant. All in vitro experiments were repeated at least three times.

Results

Cancer cell-secreted growth factors mediate the activation of the PI3K/AKT signaling pathway and the induction of cell proliferation by Id1

We investigated the involvement of autocrine mechanisms in the induction of PI3K/AKT by Id1. We found that the HKESC-3-Id1 conditioned medium (CM) induced protein expression of p-AKT and cell proliferation in the parental HKESC-3 cells; the AKT activation was attenuated when the proteins in the conditioned medium were denatured (Fig. 1A and B). These results suggest that Id1-overexpressing ESCC cells had increased secretion of growth factor(s) that served as autocrine stimulator of the PI3K/AKT pathway and cancer progression.
Id1 induces IGF-II expression and secretion

We performed antibody array-based screening to identify growth factors that were differentially secreted by Id1-overexpressing ESCC cells. The results showed a higher concentration of IGF-II in the HKESC-3-Id1 CM than in the HKESC-3-CON CM (Fig. 1C and Supplementary Fig. S1).

Western blot analysis of the cell lysates and conditioned medium showed increased IGF-II expression and secretion in three different Id1-overexpressing ESCC cell lines (Fig. 1D), and this effect was successfully abrogated by shIGF-II (Fig. 1E and F). We also performed transient transfection experiments to confirm that Id1 induced the production of IGF-II.

Figure 1. Identification of the growth factors secreted from Id1-overexpressing cells. A, the HKESC-3 cells treated with the conditioned medium (CM) from HKESC-3-Id1 (with and without heat inactivation), and HKESC-3-CON cells for 1 hour were harvested for Western blot analysis. B, HKESC-3 cells were incubated for up to 72 hours in conditioned medium from HKESC-3-CON and HKESC-3-Id1, and their growth rates determined using MTT assay. C, growth factor antibody array was incubated with the serum-free conditioned medium from HKESC-3-Id1 and HKESC-3-CON cells. The representative IGF-II and internal control (IgG) spots showed higher concentration of IGF-II in the HKESC-3-Id1 conditioned medium. D, Western blot analysis of IGF-II in the cell lysates and conditioned medium obtained from three Id1-overexpressing ESCC cell lines and the respective vector control cell lines which had been kept in serum-free medium for 24 hours. E and F, KYSE150-CON-shCON, KYSE150-Id1-shCON, and KYSE150-Id1-shIGF-II cells were compared for the expression level of IGF-II in cell lysate (E) and the conditioned medium (F) by Western blot and ELISA, respectively. G, Western blot analysis of IGF-II in cell lysates and conditioned medium of KYSE150 cells transfected with different doses of pcDNA3-Id1 expression vector or siRNA against Id1. **, P < 0.01; *** P < 0.001 compared with control.
and secretion of IGF-II in a dose-dependent manner, and that siRNA knockdown of Id1 reduced IGF-II expression (Fig. 1G). To exclude the off-target effect of siRNA, a second RNA interference sequence was used to confirm the effects of Id1 knockdown on IGF-II expression (Supplementary Fig. S2). The induction of IGF-II by Id1 was further confirmed using a panel of human cancer cell lines, including gastric, colon, liver, and prostate cancer (Supplementary Fig. S3). Furthermore, manipulation of Id1 expression in ESCC cells via overexpression or short hairpin RNA knockdown resulted in corresponding changes in expression of IGF-II mRNA (Supplementary Fig. S4), which indicate that Id1 regulates IGF-II at mRNA level.

IGF-II expression is positively correlated with Id1 and p-AKT expressions in human esophageal tumors

Immunohistochemical results in TMA showed that among the 21 of 35 cancer specimens showing upregulation (i.e., moderate to strong immunostaining) of IGF-II, 15 and 14 cases had upregulated Id1 and p-AKT expression, respectively (Supplementary Fig. S5 and Supplementary Table S1). Spearman rank correlation coefficient analysis indicated a positive correlation between IGF-II and Id1 expression (r = 0.461, P < 0.005), and between IGF-II and p-AKT (r = 0.523, P < 0.005) in the ESCC specimens. These results suggest that IGF-II expression is associated with upregulation of Id1 and p-AKT in human esophageal cancer.

Autocrine IGF-II mediates the tumor- and metastasis-promoting functions of Id1 through activation of the PI3K/AKT pathway

To determine the significance and oncogenic role of Id1-induced IGF-II in ESCC cells, neutralizing antibody against IGF-II was added to the conditioned medium from HKESC-3-Id1 cells to block the functions of IGF-II. The results showed that this treatment not only attenuated the stimulation of the PI3K/AKT pathway (Fig. 2A), but also decreased the proliferative (Fig. 2B), migratory (Fig. 2C), and survival (Fig. 2D) capabilities of HKESC-3-Id1 cells. We also studied the effects of cancer cell-derived IGF-II on anchorage-independent growth using soft agar assay. As shown in Fig. 2E, the KYSE150 cancer cells were found to have significantly enhanced colony formation ability when suspended in a mixture of agar and KYSE150-Id1-shCON conditioned medium and plated on top of the base agar layer. Moreover, in the cell invasion assay (Fig. 2F), parental T.Tn and KYSE150 cells showed higher invasive ability when suspended in the conditioned media of corresponding Id1-overexpressing transfectants (i.e., T.Tn-Id1 and KYSE150-Id1-shCON). The effects on colony formation and cell invasion were abolished by knockdown of IGF-II in Id1-overexpressing cells using shIGF-II or neutralization of IGF-II in the conditioned medium (Fig. 2E and F).

Furthermore, we found that treatment with the conditioned medium from KYSE150 cells, which had higher endogenous Id1 expression (see Supplementary Fig. S6 showing endogenous Id1 and IGF-II expression levels in ESCC cell lines), enhanced the ability of HKESC-3 cells to proliferate, migrate, and survive; the effects were abolished when endogenous Id1 expression in KYSE150 cells was inhibited by siRNA against Id1 (Supplementary Fig. S7).

The autocrine function of Id1-induced IGF-II in ESCC was also investigated in vivo. As shown in Fig. 3A, KYSE150-Id1-shCON cells produced significantly larger tumors than the KYSE150-CON-shCON cells in nude mice (742.8 ± 142.9 mm³ vs. 516.5 ± 101.8 mm³ on day 35; P < 0.05), but the stimulatory effect of Id1 on tumor growth was counteracted in the IGF-II knockdown group. Comparison of the tumor xenografts indicated the larger tumor size of the KYSE150-Id1-shCON group was due to increased cell proliferation and reduced apoptosis (Fig. 3B and Supplementary Fig. S8). Western blot analysis of the tumor xenografts showed that these changes were associated with the higher expression levels of IGF-II and p-AKT in the KYSE150-Id1-shCON tumors (Fig. 3C). Moreover, the mice bearing KYSE150-Id1-shCON xenografts had significantly higher concentration of human IGF-II in the circulation compared with the other two groups (Fig. 3D), which suggests that IGF-II may function as an endocrine growth stimulator for esophageal cancer. Our results also showed that shIGF-II successfully downregulated tumor and circulating IGF-II levels in vivo, thus abolishing the activation of the PI3K/AKT pathway by Id1 (Fig. 3C and D). We also determined the contribution of IGF-II as a mediator of Id1-induced tumor metastasis using experimental metastasis assay. KYSE150-CON-shCON, KYSE150-Id1-shCON, or KYSE150-Id1-shIGF-II cells were intravenously injected into nude mice through the tail vein. Six weeks later, numerous large metastatic nodules that showed positive immunostaining for human-specific cytokeratin-8 were found in the lungs of the KYSE150-Id1-shCON group, but not in the KYSE150-CON-shCON and KYSE150-Id1-shIGF-II groups (Fig. 3E). These results therefore support that IGF-II plays a significant role in mediating the Id1-promoted metastasis of human esophageal cancer in vivo.
Figure 3. Effects of Id1-induced IGF-II on tumorigenicity and metastasis of esophageal cancer cells in nude mice. A, growth curves of subcutaneous tumors formed by KYSE150-CON-shCON, KYSE150-Id1-shCON or KYSE150-Id1-shIGF-II cells in nude mice (n = 8 per group). B, Ki-67 proliferation and apoptosis indices of tumor xenografts. C, Western blot analysis confirmed elevated IGF-II protein levels in the KYSE150-Id1-shCON tumor xenografts, and successful knockdown of IGF-II in the KYSE-Id1-shIGF-II xenografts. D, serum IGF-II concentration was assessed by ELISA. E, KYSE150-CON-shCON, KYSE150-Id1-shCON, or KYSE150-Id1-shIGF-II cells were intravenously injected into nude mice through the tail vein (n = 8 per group). Top, representative images of lungs harvested 6 weeks postinjection (metastatic nodules are indicated by arrows). Surface metastatic nodules in the lungs were counted, and the numbers presented separately for nodules below and above 1 mm in diameter. A human-specific anti-cytokeratin-8 (CK-8) monoclonal antibody was used to distinguish human ESCC cells from the surrounding mouse pulmonary tissue (middle). Bottom, consecutive lung sections stained with hematoxylin and eosin (H&E). *, P < 0.05; **, P < 0.01; *** P < 0.001 compared with control group inoculated with KYSE150-CON-shCON cells.
Tumor-secreted IGF-II instigates growth and metastasis of distant tumor cells in an endocrine manner

Because the mice bearing Id1-expressing tumor xenografts showed elevated serum IGF-II (Fig. 3D), and exposure to exogenous IGF-II-containing culture media enhanced the in vitro tumorigenic and invasive potentials of esophageal cancer cells (Fig. 2E and F), we reasoned that, in addition to autocrine stimulation, tumor-derived IGF-II may also exert systemic endocrine effects that facilitate tumor growth at a distant site. To test this hypothesis, we constructed an in vivo experimental model (Fig. 4A) similar to that used by Weinberg's team in which more aggressive "instigator" cancer cells were injected into one flank of the experimental animal and less aggressive "responder" cells into the contralateral flank (21). KYSE150-CON-shCON, KYSE150-Id1-shCON, and KYSE150-Id1-shIGF-II cells were subcutaneously injected into the left flanks of different groups of nude mice to compare their ability to instigate tumor formation of parental KYSE150 cells inoculated subcutaneously into the right flank 2 weeks later (i.e., responder). As shown in Fig. 4B, the KYSE150-Id1-shCON group developed significantly larger responding tumors than the KYSE150-CON-shCON group, but knockdown of IGF-II in the instigating tumor attenuated the growth of responding tumor to a level comparable with that of the empty vector control group. We also found increased p-AKT expression (Fig. 4C), as well as significantly higher Ki-67 proliferation and lower apoptotic indices in the responding tumors of the KYSE150-Id1-shCON group compared with the KYSE150-Id1-shIGF-II and KYSE150-CON-shCON groups (Fig. 4D and Supplementary Fig. S9), suggesting that IGF-II secreted by primary tumors can exert endocrine influence to instigate the growth of less aggressive tumors at a distant site through activation of the PI3K/AKT signaling pathway (Fig. 4E).

To determine whether endocrine IGF-II can facilitate the outgrowth of disseminated tumor cells, a modified experimental metastasis assay was performed in which parental KYSE150 cells were injected intravenously into the tail vein of nude mice which were inoculated 2 weeks earlier with KYSE150-CON-shCON, KYSE150-Id1-shCON, or KYSE150-Id1-shIGF-II subcutaneous xenografts. Six weeks later, we found more metastatic nodules in the lungs of the KYSE150-Id1-shCON group, than the other groups (Fig. 4F). These findings demonstrated that Id1-overexpressing tumors can facilitate metastatic colonization of disseminated cancer cells by secreting IGF-II. This aspect may have important therapeutic implications for cancer treatment.

IGF-II neutralization suppresses esophageal cancer growth in vivo

To investigate the antitumor effects of IGF-II-neutralization on esophageal cancer, nude mice were inoculated with KYSE150 cells and given intratumoral injection of IGF-II-neutralizing antibody. As shown in Fig. 5A, treatment with IGF-II-neutralizing antibody for 15 days significantly suppressed tumor growth by approximately 49% without detectable toxic effects on body weight, the lungs, liver, or kidneys (Supplementary Fig. S10A and S10B). Histologic evaluation showed that the suppressive effect of IGF-II-neutralizing antibody on the tumor growth was due to decreased cell proliferation rate and vascular supply, as well as increased apoptosis (Supplementary Fig. S10C).

IGF-IR blockade inhibits esophageal cancer growth and metastasis in vivo

Because the biologic functions of IGF-II are mainly exerted through IGF-IR, we next studied whether systemic blocking of IGF-IR may inhibit tumor growth in vivo. Cixutumumab, a fully human immunoglobulin G (IgG)-1 monoclonal antibody targeting human IGF-IR (22), was injected intraperitoneally into nude mice bearing ESCC tumor xenografts. We found a significant dose-dependent reduction of tumor volume 19 days after treatment, with decreases of 66.4% and 76.6% for KYSE150 and KYSE270 tumors, respectively, in the groups receiving 50 mg/kg cixutumumab treatment (Fig. 5B). Histologic comparison of the tumor xenografts indicated that cixutumumab treatment decreased the proliferation rate and enhanced apoptosis of cancer cells, as well as reduced tumor vascular supply (Fig. 5C and Supplementary Figs. S11 and S12). Notably, Western blot and immunohistochemical analysis of tumor xenografts suggested that the inhibitory effects of cixutumumab on tumor growth were due to inactivation of the PI3K/AKT pathway (Fig. 5D and E). The elevated expression of cleaved caspase-3 also confirmed the increased apoptosis in the tumor cells of cixutumumab-treated mice (Fig. 5D). Furthermore, although cixutumumab crossreacts with mouse IGF-IR (22), cixutumumab had no obvious adverse effects on the body weight and vital organs (Supplementary Fig. S13). To evaluate the treatment efficacy of IGF-IR blockade on metastasis, nude mice were intravenously injected with luciferase-expressing ESCC cells, then treated with cixutumumab. Bioluminescent imaging and histologic examination showed marked lung metastasis in the control groups but significantly less metastatic activity in the lungs of the cixutumumab-treated groups (Fig. 6A–C). On the basis of these in vivo data, we conclude that blockade of IGF-IR with cixutumumab can significantly suppress the growth of tumor and metastasis.

Cixutumumab reverts acquired chemoresistance in fluorouracil-resistant ESCC sublines and enhances the chemosensitivity of ESCC cells in vivo

Development of drug resistance often occurs in patients treated with conventional chemotherapeutic reagents. To mimic the process by which patients acquire chemoresistance, fluorouracil (5-FU)-resistant ESCC sublines were established by treating cells with increasing doses of 5-FU (up to 40 μmol/L) for over a year, and were highly resistant to 5-FU treatment (Fig. 6D). Our results showed that the fluorouracil-resistant cells had higher expression of thymidylate synthase than the parental cells (Supplementary Fig. S14A), and that cixutumumab treatment significantly...
reduced the expressions of thymidylate synthase and p-AKT in the fluorouracil-resistant cells (Supplementary Fig. S14B). Cixutumumab alone could moderately inhibit the proliferation of the fluorouracil-resistant cells, and that the acquired chemoresistance in fluorouracil-resistant cells was significantly abrogated by a combination of cixutumumab and 5-FU treatment (Fig. 6E). We then proceeded to investigate the therapeutic potential of cixutumumab in combination with conventional cytotoxic chemotherapeutic drugs in vivo. Figure 6F shows that cixutumumab markedly...
augmented the sensitivity of ESCC tumors to 5-FU and cisplatin.

Discussion
In this study, we provide the first evidence that Id1 induces IGF-II in a variety of cancer cells, and that tumor-secreted IGF-II not only promotes local tumor growth through autocrine stimulation of the PI3K/AKT pathway, but also acts systemically to instigate the growth of distant tumors and facilitates the metastasis of circulating esophageal cancer cells in an endocrine manner. Furthermore, because our results showed that neutralization of IGF-II and IGF-IR blockade had significant antitumor effects,
including enhancing chemosensitivity, and inhibiting tumor growth, and metastasis in animal models, we propose targeting IGF-II or IGF-IR as a potentially promising strategy in esophageal cancer therapy.

Here, we have uncovered a novel mechanism in which Id1 activates the PI3K/AKT pathway through induction and secretion of autocrine IGF-II. Our finding that Id1 increases the expression and secretion of IGF-II is significant because to our knowledge, the only other growth factor reported to be induced by Id1 expression in cancer cells is VEGF (23, 24). However, VEGF is unlikely to account for the diverse tumor-promoting effects of Id1. Data from our and neutralization experiments strongly support IGF-II as a key growth factor that mediates the oncogenic functions of Id1.

IGF-II is overexpressed in a spectrum of human cancers, including colorectal, breast, ovarian, testicular, and liver cancer (25–29). An earlier immunohistochemical study showed that 50% of ESCC tumors express IGF-II which is associated with disease progression and recurrence (13). Incidentally, our TMA analysis showed a positive correlation between Id1 and IGF-II expressions in esophageal cancer cells.

We used xenograft models to evaluate the role of IGF-II in the development of esophageal cancer and chemoresistance. Treatment of the mice with 25 or 50 mg/kg cixutumumab twice a week significantly reduced the extent of lung metastasis (n = 6 per group). Representative photos of bioluminescent imaging of the mice 8 weeks after cell injection were shown here. Bioluminescent signals detected in the region of the lungs of the mice from different treatment groups were analyzed. C, representative hematoxylin and eosin (H&E)-stained lung sections from the three groups of mice. D, MTT assay comparing the viability of fluorouracil-resistant (FR) ESCC sublines with that of parental cells 4 days after treatment with 5-FU (20 μmol/L for KYSE150 cell lines, 10 μmol/L for KYSE410 cell lines, and 5 μmol/L for KYSE270 cell lines). E, FR cells were treated with 5-FU, alone or in combination with cixutumumab (100 μg/mL), and cell viability compared using MTT assay. F, nude mice xenografted with human ESCC cells KYSE150 (left) and KYSE270 (right) were treated with cixutumumab (25 mg/kg), 5-FU (20 mg/kg), cisplatin (2 mg/kg), cixutumumab plus 5-FU, or cixutumumab plus cisplatin twice weekly (n = 6 per group). Bars, SD; ***, P < 0.001 compared with control.
tumors. It was proposed that growth and metastasis of tumors can be governed on a systemic level by endocrine factors released by tumors at distant sites (21). By demonstrating that nude mice bearing Id1-expressing tumor xenografts had increased serum IGF-II, which exerted a systemic instigating effect on tumor growth at distant sites, we have provided new evidence to support this concept. Furthermore, our results also indicated that tumor-secreted IGF-II could promote the colonization and proliferation of blood-borne ESCC cells at secondary sites. These findings have important implications in controlling cancer progression. In previous studies, including our own, the role of Id1 in metastasis was thought to be limited to enhancing cell migration, cell invasion, epithelial to mesenchymal transition, and production of matrix metalloproteinase, which are the key initial steps of metastasis (19, 30, 31). Here, we further demonstrated that Id1 plays an important role in the final steps of the metastatic cascade by increasing circulating IGF-II.

Esophageal cancer is a highly lethal malignancy, and development of new therapeutic intervention is urgently needed. Anticancer reagents using neutralizing antibodies to target growth factor and their receptors have emerged as a new class of effective therapeutics for human cancer. Because previous studies showed that as high as 60% of ESCC tumors expresses IGF-IR (13), inhibition of the IGF signaling pathway was proposed as a potential therapeutic option for ESCC. However, very little has been reported about the efficacy of IGF-II targeting in preclinical studies or clinical trials. Our current study provides the first evidence that neutralizing IGF-II alone can significantly inhibit the growth of human ESCC tumor xenografts. A number of IGF-IR inhibitors are currently in clinical trials for the treatment of solid tumors (32). We found that cixutumumab not only had significant inhibitory effects on esophageal cancer growth and metastasis, but also markedly enhanced the sensitivity of cancer cells to chemotherapeutics and to target growth factor and their receptors have emerged as a new class of effective therapeutics for human cancer. Because previous studies showed that as high as 60% of ESCC tumors expresses IGF-IR (13), inhibition of the IGF signaling pathway was proposed as a potential therapeutic option for ESCC. However, very little has been reported about the efficacy of IGF-II targeting in preclinical studies or clinical trials. Our current study provides the first evidence that neutralizing IGF-II alone can significantly inhibit the growth of human ESCC tumor xenografts. A number of IGF-IR inhibitors are currently in clinical trials for the treatment of solid tumors (32). We found that cixutumumab not only had significant inhibitory effects on esophageal cancer growth and metastasis, but also markedly enhanced the sensitivity of cancer cells to chemotherapeutics.

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