

Insulin-like growth factor-I receptor blockade reduces the invasiveness of gastrointestinal cancers via blocking production of matrilysin

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Insulin-like growth factor-I receptor (IGF-IR) signaling is required for carcinogenicity and proliferation of gastrointestinal (GI) cancers. We have previously shown significant therapeutic activity for recombinant adenoviruses expressing dominant-negative insulin-like growth factor-I receptor (IGF-IR/dn), including suppression of tumor invasion. In this study, we sought to evaluate the mechanism of inhibition of invasion and the relationship between IGF-IR and matrix metalloproteinase (MMP) activity in GI carcinomas. We analyzed the role of IGF-IR on invasion in three GI cancer cell lines, colorectal adenocarcinoma, HT29; pancreatic adenocarcinoma, BxPC3 and gastric adenocarcinoma, MKN45, using a modified Boyden chamber method and subcutaneous xenografts in nude mice. The impact of IGF-IR signaling on the expression of MMPs and the effects of blockade of matrilysin or IGF-IR on invasiveness were assessed using recombinant adenoviruses, a tyrosine kinase inhibitor NVP-AEW541 and antisense matrilysin. Invasive subcutaneous tumors expressed several MMPs. IGF-IR/dn reduced the expression of these MMPs but especially matrilysin (MMP-7). Insulin-like growth factor (IGF) stimulated secretion of matrilysin and IGF-IR/dn blocked IGF-mediated matrilysin induction in three GI cancers. Both IGF-IR/dn and inhibition of matrilysin reduced *in vitro* invasion to the same degree. NVP-AEW541 also reduced cancer cell invasion both *in vitro* and in murine xenograft tumors via suppression of matrilysin. Thus, blockade of IGF-IR is involved in the suppression of cancer cell invasion through downregulation of matrilysin. Strategies of targeting IGF-IR may have significant therapeutic utility to prevent invasion and progression of human GI carcinomas.

Introduction

Signals from a variety of growth factor receptors are required for carcinogenesis and tumor development in human neoplasms, including gastrointestinal (GI) carcinomas. These signals alter cell cycle regulation, induction of apoptosis and interactions of cancer cells with their environment to promote the continuous growth potential of tumor cells (1). Insulin-like growth factor-I receptor (IGF-IR) is a heterodimer of two α -subunits (containing the extracellular domain) and two β -subunits (with transmembrane and tyrosine kinase domains)

Abbreviations: GI, gastrointestinal; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein; IGF-IR, insulin-like growth factor-I receptor; IGF-IR/dn, dominant-negative insulin-like growth factor-I receptor; MMP, matrix metalloproteinase; PI3-K, phosphatidylinositol 3-kinase; TIMP, tissue inhibitor of metalloproteinase; TKI, tyrosine kinase inhibitor.

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(2). Binding of the ligands, insulin-like growth factor (IGF)-I and IGF-II, to IGF-IR cause receptor autophosphorylation and activate multiple signaling pathways, including the mitogen-activated protein kinase/extracellular signal-regulated kinase and phosphatidylinositol 3-kinase (PI3-K)/Akt-1 pathways (3). In normal cells, activation of IGF-IR is tightly regulated by the action of insulin-like growth factor-binding proteins (IGFBP) and the non-stimulatory receptor, type 2 IGF receptor (IGF-IIR) (4–7).

Dysregulation of the IGF-I system has been implicated in the proliferation of numerous malignancies (7,8). Recent studies have shown that elevation of serum IGF-I increases the risk of developing several cancers, including colon (5). IGF-IR is important for tumor maintenance as well as malignant transformation (9,10). Reduction of IGF-IR has been shown to induce apoptosis in tumors, but produce only growth arrest in untransformed cells (1), implying that receptor blockade may have a greater therapeutic index than other strategies targeting fundamental cell mechanisms such as DNA synthesis. Additionally, IGF-IR knockout mice are viable indicating that relatively normal tissue development and differentiation occurs in the absence of IGF-IR (11).

Exogenous IGFs stimulate the proliferation of GI cancer cells and blocking of IGF-IR signaling inhibits tumor growth (4,12–14). Intestinal fibroblast-derived IGF-II also stimulates proliferation of intestinal epithelial cells in a paracrine manner (15). Soluble IGF-IIR rescues Apc (Min/+) intestinal adenoma progression induced by loss of IGF-II imprinting (6). Expression of both IGF-II and IGF-IR are increased in GI cancers (12,16). IGF-II, in conjunction with IGF-IR, IGF-I, cyclooxygenase-2 and MMP-7, seems to play a key role in the early stage of colorectal carcinogenesis (17,18). Moreover, IGF-IR signaling is important in tumor dissemination through the control of adhesion, migration, invasion and metastasis. These findings suggest a potential basis for tumor selectivity in therapeutic applications in GI cancers.

One of the most important mechanisms in tumor invasion is proteolytic degradation of extracellular matrix (19). Matrix metalloproteinases (MMPs) have been implicated in tumor invasion as well as in various normal processes of tissue remodeling (20,21). Moreover, MMPs are often overexpressed during tumor progression. Matrilysin (MMP-7) is a member of the MMP gene family and is distinguished from other MMPs by some unique characteristics: a broad proteolytic activity against a variety of extracellular matrix substrates; the lowest molecular weight in the family; a potency to start an activation cascade of MMPs and production by cancer cells but not stromal cells (22–24). Matrilysin has been detected in multiple tumor cell types in addition to several normal tissues (24–27). In colorectal cancer, matrilysin plays roles not only in tumor progression but also in carcinogenesis (28). We have previously reported that matrilysin is overexpressed in colorectal cancers and its expression increases with advancing stage (24,25). We have also demonstrated that the transfection of its complementary DNA caused colon cancer cells to be more invasive and more metastatic (29,30). In contrast, downregulation of this proteinase by introduction of antisense matrilysin complementary DNA or all-trans retinoic acid made these cells less invasive *in vitro* (29,31).

Recently, advances in molecular research in cancer have brought new therapeutic strategies into clinical usage. Receptor tyrosine kinases are one new group of targets and can be blocked by small molecule tyrosine kinase inhibitors (TKI) or monoclonal antibodies. IGF-IR may be a promising new molecular target in many human malignancies (7,8). Several possible approaches to block IGF-IR signaling have been reported. Monoclonal antibodies for IGF-IR are in phase I and II trials (32). Small molecular TKI for IGF-IR have been developed, including NVP-AEW541 (33). The orally available compound NVP-AEW541 is a novel small molecule

IGF-IR kinase inhibitor and is a pyrrolo[2,3-d]pyrimidine derivative (33). It is highly selective against IGF-IR, compared with the insulin receptor (IR) and other tyrosine kinases, and has significant therapeutic utility in human GI carcinomas both alone and in combination with chemotherapy (34). We have also constructed two dominant-negative inhibitors for IGF-IR [dominant-negative insulin-like growth factor-I receptor (IGF-IR/dn); IGF-IR/482st and IGF-IR/950st], which are active as plasmids and recombinant adenovirus vectors in GI malignancies, including colorectal, pancreatic, gastric and esophageal cancers (35–38). We have reported that both agents that blockade IGF-IR, IGF-IR/dn and AEW541 suppressed carcinogenicity and upregulated stressor- or chemotherapy-induced apoptosis in GI carcinomas, both *in vitro* and *in vivo*. Moreover, we have found that IGF-IR/dn reduce invasiveness of xenografted tumors in nude mice (36,37).

In this study, we analyzed the impact and mechanism of IGF-IR on tumor invasiveness. We also assessed the effectiveness of IGF-IR blockade on reducing cancer invasion and MMP expression. In addition to the established effects of IGF-IR blockade on apoptosis, these results establish direct involvement of this receptor in tumor invasiveness and strengthen the rationale for using IGF-IR blockade in the molecular targeted therapy of GI cancers.

Materials and methods

Materials, cell lines and mice

Anti-Akt1(c-20), anti-IGF-I (G-17), anti-IGF-IR α (2C8), anti-MMP2 (C19) and anti-MMP9 (C20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-phospho-Akt(Ser473) and PathScan Multiplex Western Cocktail I were from Cell Signaling Technology (Beverly, MA). Monoclonal antibodies for MMP-1 (41-1E5), -3 (55-2A4) and -7 (141-7B2) were purchased from Fuji Chemical (Toyama, Japan). Recombinant human IGF-I and IGF-II were purchased from R&D systems (Minneapolis, MN). All human GI cancer cell lines, colorectal adenocarcinoma cells, HT29 and SW480, gastric adenocarcinoma cells, MKN45 and NUGC4 and pancreatic adenocarcinoma cell, BxPC3, were obtained from Japanese Cancer Collection of Research Biorepositories Cell Bank (Tokyo, Japan). Cells were passaged in RPMI1640 and Dulbecco's modified Eagle's medium both with 10% fetal bovine serum. Specific pathogen-free female BALB/cAnNCrj-nu mice, 6-weeks-old, were purchased from Charles River (Yokohama, Japan). The care and use of mice were according to our university's guidelines.

NVP-AEW541 was kindly provided by Novartis Pharma (Basel, Switzerland). Stock solution of this drug was prepared in dimethyl sulfoxide and stored at -20°C .

Reverse transcription–polymerase chain reaction

Total RNA from cells was isolated by the acid guanidinium thiocyanate–phenol–chloroform method. The complementary DNAs were synthesized from 1 μg of total RNA by M-MLV reverse transcriptase (Takara, Japan) and amplified by polymerase chain reaction using MMP-2, -3, -7 and -9, and GAPDH-specific oligonucleotides synthesized on the basis of the sequence reported previously (39); sense 5'-CCACGTGACAAG CCCATGGGGCCCC-3' and antisense 5'-GCAGCCTAGCCAGTCGGATTTGATG-3' for MMP-2; sense 5'-GGTCCCCCCTGCTGGCCCTTCTACGGCC-3' and antisense 5'-GTCCTCAGGGCACTGCAGGATGTCATAGGT-3' for MMP-9; sense 5'-AGATGTGGAGTGCCAGATGT-3' and antisense 5'-TAGACTGCTAC-CATCCGTCC-3' for matrilysin and sense 5'-CAGCCGAGCCACATCG-3' and antisense 5'-TGAGGCTGTTGTCATACTTCTC-3' for GAPDH. GAPDH served as an internal control of the reaction. Polymerase chain reaction was carried out for one cycle at 94°C for 4 min followed by 20–30 cycles at 94°C for 30 s, 58°C for 30 s and 72°C for 3 min.

Western blotting and immunoprecipitation

Cells were treated as indicated in the text. Cell lysates were prepared as described previously (35). Equal aliquots of lysate (100 μg) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotted onto polyvinylidene Hybond-P membrane (Amersham, Arlington Heights, IL). Analysis was performed using indicated antibodies, and bands were visualized by Enhanced Chemiluminescence (Amersham).

To analyze IGF-IR or IR signaling, 1 mg of whole-cell lysates were immunoprecipitated overnight at 4°C with anti-IGF-IR or anti-IR antibodies followed by western blotting. The desired proteins were detected by the indicated antibodies.

Zymographic analysis

Zymography was performed as described previously (26). Zymography in sodium dodecyl sulfate–polyacrylamide gel electrophoresis containing casein was performed for matrilysin and in gels containing gelatin for gelatinases. Equal amounts of protein were electrophoresed in a 10% polyacrylamide gel embedded with 1 mg/ml casein or gelatin. After electrophoresis, the gels were washed in 2.5% Triton X-100 for 1 h to remove sodium dodecyl sulfate. The gels were then incubated for 18 h at 37°C in 50 mM Tris–HCl (pH 7.5), 10 mM CaCl_2 and 0.02% NaN_3 , stained with Coomassie Brilliant Blue and then destained.

In vivo therapeutic efficacy in established tumors

We evaluated the effect of either ad-IGF-IR/482st or NVP-AEW541 of established tumors. A total of 1×10^6 BxPC3 or MKN45 were subcutaneously injected into nude mice. After forming subcutaneous tumors on nude mice, we treated those tumors with intratumor injection of ad-IGF-IR/482st or ad-LacZ on five successive days. In another group, HT29 or BxPC3 tumor-bearing nude mice were treated per os twice daily for 7 days either with NVP-AEW541 (40 mg/kg; 10 ml/kg dissolved in 25 mM L-(+)-tartaric acid, therapy group) or with 25 mM L-(+)-tartaric acid (control group). Three days after completing this treatment, mice were killed.

Immunohistochemical analysis

Sections (5 μm) from formalin-fixed, paraffin-embedded tumor xenografts were prepared. After deparaffinization, the sections were pretreated with DakoCytomation Target Retrieval Solution (Dako, Glostrup, Denmark) in a microwave (10 min). Then, endogenous peroxidase activity was blocked. Antibodies were applied after blocking with normal goat serum. Sections were incubated with the anti-rabbit secondary antibody (Santa Cruz Biotechnology) and streptavidin–horseradish peroxidase (Dako) followed by exposure to the diaminobenzidine tetrahydrochloride substrate (Dako).

Measurement of the immunostained area in pictures was performed using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>).

In vitro invasion assay

The assay was performed by a modified Boyden Chamber method as described previously (26). First, GI cancer cell lines were infected with ad-IGF-IR/482st or ad-lacZ. A total of 2×10^5 cells were seeded on Matrigel-coated filters (8 μm diameter pores, 100 μg per filter; Beckton Dickinson, Bedford, MA). The cells on the upper surface of the filters were completely removed after 24 or 48 h incubation with or without tissue inhibitor of metalloproteinases (TIMPs). In other wells, 1 ng of matrilysin antisense oligonucleotides (5'-GTATATGATACGATC-3', MMP-7 AS, Calbiochem, San Diego, CA) or control (5'-GTATTAGTATCGAAC-3', Calbiochem) was added to each well and then HT29 was cultured. Non-infected HT29 was cultured with or without the TKI. The filters were fixed with cold methanol and stained with hematoxylin and eosin. The cells that had invaded to the lower side of the filters were counted under a microscope by two independent observers.

Statistical analysis

The results are presented as means \pm SEs for each sample. The statistical significance of differences was determined by Student's two-tailed *t*-test in two groups and done by one-way analysis of variance in multiple groups and two-factor factorial analysis of variance. *P* values of <0.05 were considered to indicate statistical significance.

Results

The expression of MMP message in subcutaneous xenografts

In order to investigate the role of MMPs in the previously reported IGF-IR/dn-mediated suppression of invasion (Figure 1A) (36), the expression of MMP messenger RNAs was analyzed by reverse transcription–polymerase chain reaction (data not shown). All BxPC3 tumors treated with control adenovirus produced MMP-2 and MMP-9, 83% tumors expressed MMP-3 and 67% tumors expressed matrilysin. Although BxPC3 tumors treated with IGF-IR/dn expressed MMP-9 in all and MMP-2 and -3 in 83%, matrilysin was suppressed in all. This result indicates that IGF-IR/dn reduces the expression of key MMPs, particularly matrilysin, that in turn may underlie the observed reduced invasive potential of these tumors.

In our previous study (36), mice were killed over 1 month after treatment. In order to analyze MMP expression acutely regulated by IGF-IR blockade, we did the following experiments. After

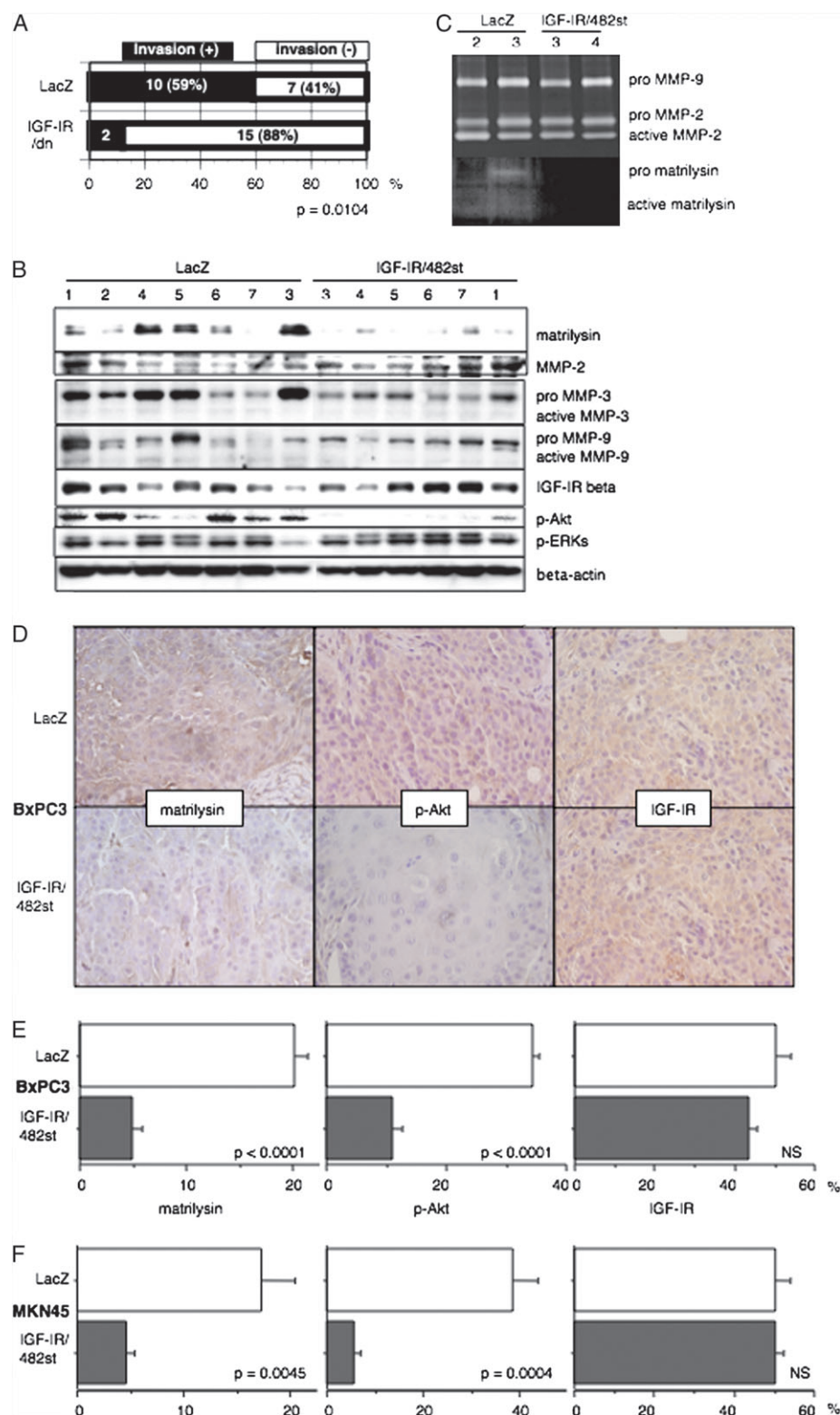


Fig. 1. The invasiveness of subcutaneous tumors on nude mice. (A) The invasion to the underlying muscle layer in BxPC3 SC tumors were detected 1 month after treatment with IGF-IR/dn. The number of invasive tumor treated with IGF-IR/dn was less than that with control ($P = 0.0104$, Fisher's exact test). (B–E) Subcutaneous BxPC3 tumors were analyzed 3 days after treatment with IGF-IR/dn. (B) Western blot analysis shows that matrilysin expression, but not most of the other MMPs, was downregulated and Akt was hypo-phosphorylated after treatment with IGF-IR/dn. (C) Gelatin zymography shows that IGF-IR/dn did not affect the activities of MMP-2 and -9. Casein zymography revealed that matrilysin activity was not detected after treatment by IGF-IR/dn. (D) Anti-matrilysin(141-7B2) and anti-phospho-Akt(Ser473) were used for immunohistochemical analysis ($\times 400$). The expression of matrilysin in the BxPC3 tumors treated with IGF-IR/482st was reduced compared to that of control. Akt was more phosphorylated in BxPC3 tumors treated with control than IGF-IR/482st. IGF-IR expression was not influenced by IGF-IR/482st. (E and F) The immunohistochemically positive area was quantitatively measured using NIH image software. In both BxPC3 (E) and MKN45 (F) tumors, both matrilysin expression and Akt activation were reduced by treatment with IGF-IR/dn but IGF-IR expression was not. Western blots are representative of one of triplicate experiments. Representative panels of zymography are one of two or more experiments.

subcutaneous tumors were allowed to form in nude mice, we treated these tumors with ad-IGF-IR/482st or ad-LacZ for five successive days. Three days after completion of this treatment, mice were killed. Most BxPC3 tumors treated with control expressed IGF-IR and MMPs-2, -3, -7 and -9 and both Akt-1 and extracellular signal-regulated kinases were phosphorylated (Figure 1B). IGF-IR expression in BxPC3 tumors treated with IGF-IR/dn was not different from that observed with control virus. Although all BxPC3 treated with IGF-IR/482st secreted MMPs-2, -3 and -9, the production of matrilysin was less frequent and at a lower level than that observed in the LacZ control. Although the extracellular signal-regulated kinases were phosphorylated, phospho Akt was detected in only one of the tumors treated with IGF-IR/dn. Zymography confirmed the observation that matrilysin was not detected in BxPC3 tumors treated with IGF-IR/482st, although both MMP-2 and MMP-9 were not different between the two treatment groups (Figure 1C). Immunohistochemical analysis also confirmed that IGF-IR/dn reduced both matrilysin expression and phosphorylated Akt-1 (Figure 1D). Quantitation of the intensity of staining in immunohistochemically positive areas using NIH image software confirmed that IGF-IR/dn inhibited both matrilysin expression and activation of Akt without influencing IGF-IR expression (Figure 1E). In another experiment, both matrilysin expression and phosphorylation of Akt were clearly reduced in subcutaneous MKN45 tumors treated with IGF-IR/dn without changes in the expression levels of IGF-IR (Figure 1F). Those results demonstrate that Akt pathway activation is highly correlated with matrilysin regulation by IGF-IR/dn.

IGF stimulates matrilysin expression

To assess the effects of IGF on *in vitro* matrilysin expression in GI cancer cell lines, we performed western blot assays (Figure 2A). A 100 ng/ml IGF-I significantly stimulates matrilysin expression between 8 and 48 h in BxPC3 cells. IGF-I upregulated matrilysin expression in HT29 cells for 24 h. IGF-I also enhanced matrilysin secretion in MKN45 cells. Lower concentrations of IGF-I (10 ng/ml) also upregulated matrilysin expression in BxPC3 cells (Figure 2B). However, IGF-I did not influence the activity of MMP-2 and MMP-9 in BxPC3 detected by gelatin zymography (Figure 2C). IGF-I also did not affect on expressions of MMP-2, -3 and -9 in HT29 (Figure 2C).

The effect of IGF-IR/dn on MMP expression in GI cancer cells

In order to evaluate the effect of IGF-IR/482st on IGF-induced expression of matrilysin messenger RNA, northern blot assays were

performed. Matrilysin expression was dramatically reduced by IGF-IR/dn in all three cell lines tested, HT29, MKN45 and BxPC3 (Figure 3A).

Then, we assessed the effect of IGF-IR/dn on MMP protein expression and proteolytic activity using western blot assays and zymography. In MKN45 cells, western blotting revealed IGF-I stimulated matrilysin expression and IGF-IR/482st blocked its expression (Figure 3B). However, IGF did not stimulate the expressions of other MMPs. Gelatin zymography confirmed that the expressions of pro-MMP-9, pro-MMP-2 and active MMP-2 were not influenced by the presence of IGF-I and IGF-IR/dn. Casein zymography showed IGF-IR/482st blocked expression and activation of matrilysin. As we reported previously (35,36), IGF-IR/482st blocked ligand-stimulated IGF-IR signaling and phosphorylated Akt-1 (Figure 3C). PI3-K inhibitor, Wortmannin, downregulated IGF-induced matrilysin message (Figure 3D), indicating that PI3-K/Akt might play an important part in ligand-induced matrilysin expression. In HT29 cells, both IGF-I and IGF-II stimulated matrilysin expression and IGF-IR/482st blocked its expression (Figure 3E). However, both IGFs did not stimulate the expressions of other MMPs and the presence of IGF-IR/dn did not affect their levels. In BxPC3 cells, matrilysin was upregulated by IGF-I, and this was blocked by IGF-IR/dn (Figure 3F). Gelatin zymography demonstrated that both IGFs did not stimulate both MMP-2 and MMP-9 expressions, and IGF-IR/dn did not affect these activities.

Blockade of IGF-IR reduced *in vitro* invasion

To assess the effect of IGF-IR/dn on *in vitro* invasiveness, we performed *in vitro* invasion assays. MKN45 cells infected with control adenovirus readily invaded through Matrigel-coated membranes. IGF-IR/482st reduced this invasiveness by about 45% (Figure 4A). Treatment with the natural MMP inhibitors TIMP-1 and TIMP-2 blocked the invasiveness to a similar degree as IGF-IR/dn and TIMP-2 showed a dose-dependent effect. Moreover, antisense matrilysin suppressed the number of invaded cells even more than IGF-IR/dn. These results suggest that IGF-IR/dn reduced the invasiveness of MKN45 via suppression of MMPs, especially matrilysin. The decrease of invasion by IGF-IR/dn was also observed in another gastric cancer cell line, NUGC4.

The reduction of invasiveness by IGF-IR/dn was also observed in two colon cancer cell lines, HT29 and SW480 (Figure 4A and B). The effectiveness of TIMPs and antisense matrilysin in blocking invasion was similar to that seen after treatment with IGF-IR/482st, consistent

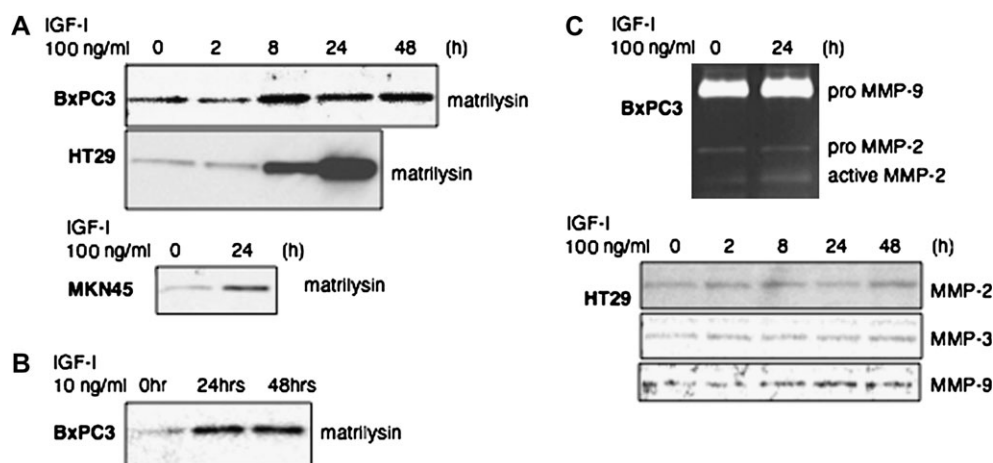


Fig. 2. IGF-I induces expression of matrilysin. (A) Western blotting shows that 100 ng/ml IGF-I stimulates matrilysin in BxPC3, HT29 and MKN45. (B) In BxPC3 cells, 10 ng/ml IGF-I induced matrilysin by western blotting. (C) In BxPC3, 100 ng/ml IGF-I did not influence on MMP-2 and -9 by gelatin zymography. In HT29, 100 ng/ml IGF-I did not affect on expressions of MMP-2, -3 and -9 by western blotting. Western blots and zymographies are representative of triplicate experiments.

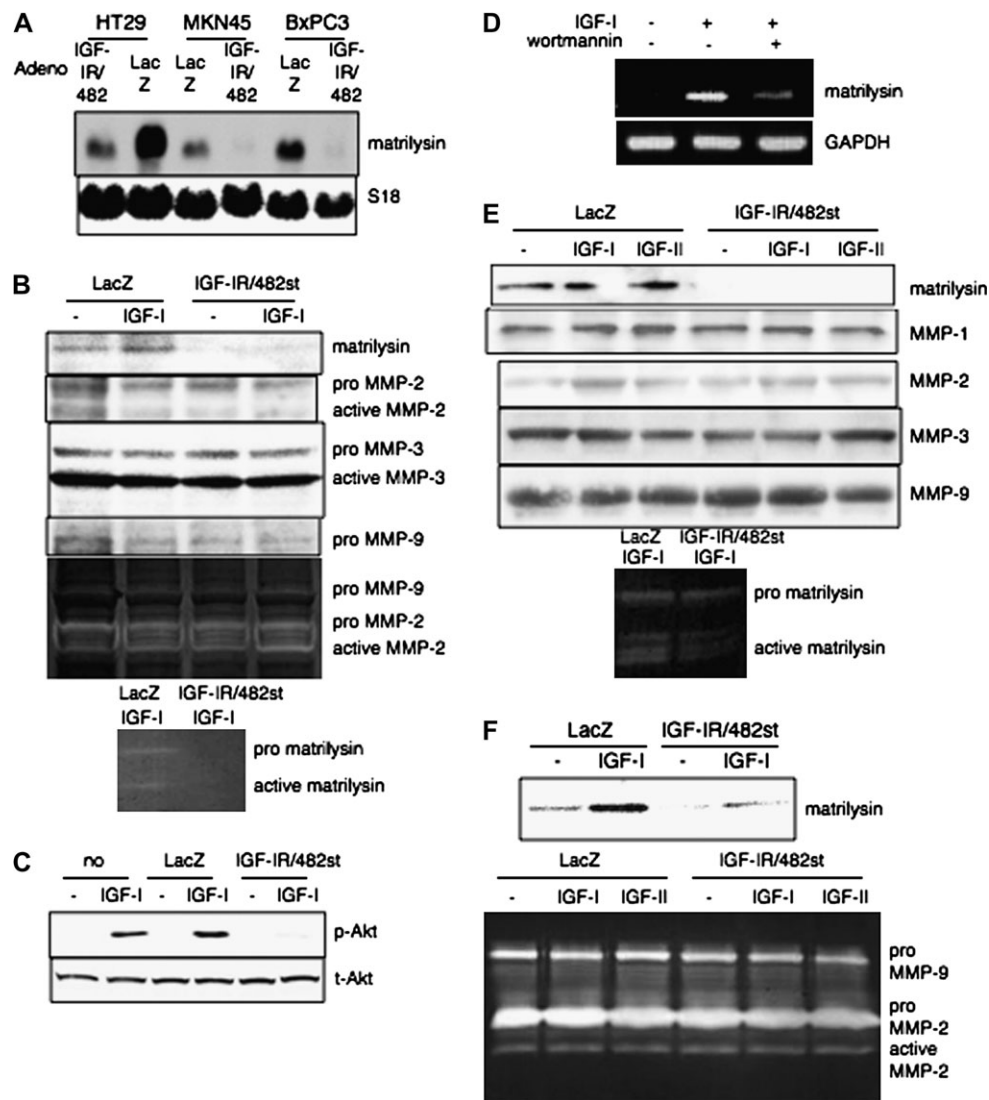


Fig. 3. IGF-IR/dn downregulates expression of matrilysin. (A) Northern blotting shows that matrilysin messenger RNA was reduced by IGF-IR/482st in three GI cancer cell lines. (B) In MKN45 cells, IGF-IR/dn blocked 10 ng/ml IGF-I-induced matrilysin expression but did not influence other MMPs, analyzed by western blotting. Gelatin zymography showed both pro and active forms of MMP2 and pro-MMP9 were not changed by IGF-IR/482st. Casein zymography revealed that IGF-IR/dn blocked expression and activation of matrilysin. (C) Western blotting shows that IGF-IR/dn blocked phosphorylation of Akt-1 in MKN45. (D) Wortmannin reduced IGF-induced matrilysin messenger RNA expression in MKN45 detected by reverse transcription–polymerase chain reaction. (E) In HT29 cells, IGF-IR/482st reduced only matrilysin protein among several MMPs detected by western blotting. Casein zymography revealed that IGF-IR/dn reduced expression and activation of matrilysin. (F) In BxPC3, western blotting showed that IGF-IR/dn reduced matrilysin secretion stimulated by 10 ng/ml IGF-I. Gelatin zymography reveals that both IGFs and IGF-IR/482st did not affect the activities of MMP-2 and -9. Western blots and zymographies are representative of triplicate experiments.

with the hypothesis that the anti-invasive effect of IGF-IR/dn works through inhibition of these MMPs, especially matrilysin. In addition to IGF-IR/482st, another dominant-negative IGF-IR/950st also reduced the number of invaded BxPC3 cell (Figure 4B).

A specific kinase inhibitor for IGF-IR NVP-AEW541 also reduced invasiveness of HT29 cells (Figure 4C). Thus, IGF-IR blockade dramatically reduced invasion and progression.

The effect of NVP-AEW541 on expression of matrilysin

To assess the impact of NVP-AEW541 on the expression of matrilysin, BxPC3 cells were cultured in serum-free media with or without AEW541 and then stimulated with IGF-I (Figure 5A). AEW541-downregulated IGF-I stimulated upregulation of matrilysin in BxPC3. However, the levels of MMP-2 and MMP-9 were not changed by IGF-I with or without AEW-541. AEW541 also reduced IGF-I-stimulated matrilysin expression in both MKN45 and HT29 (Figure 5A). As seen

in previous studies (33), AEW541 reduced IGF-I-induced phosphorylation of Akt-1 (Figure 5B). Thus, two completely different inhibitors of the same pathway, IGF-IR/dn and the tyrosine kinase inhibitor, reduced matrilysin protein in these three GI cancer cell lines, consistent with the observed effects being directly a consequence of the IGF-IR blockade and not an off-target effect of either agent.

In order to assess the effect of this drug on *in vivo* GI cancer tumors, HT29 cells were inoculated into nude mice and allowed to form evident tumors. Oral administration of 40 mg/kg NVP-AEW541 (twice a day, 2 weeks) significantly inhibited HT29 tumor invasion to the underlying muscle on mice (Figure 5C). Although the size of tumors on mice treated with AEW541 was smaller than that of control, there was no correlation between tumor size and invasiveness; in tumors <500 mm³, 0/6 demonstrated invasion with AEW541 and 2/5 showed invasion in the control; for tumors

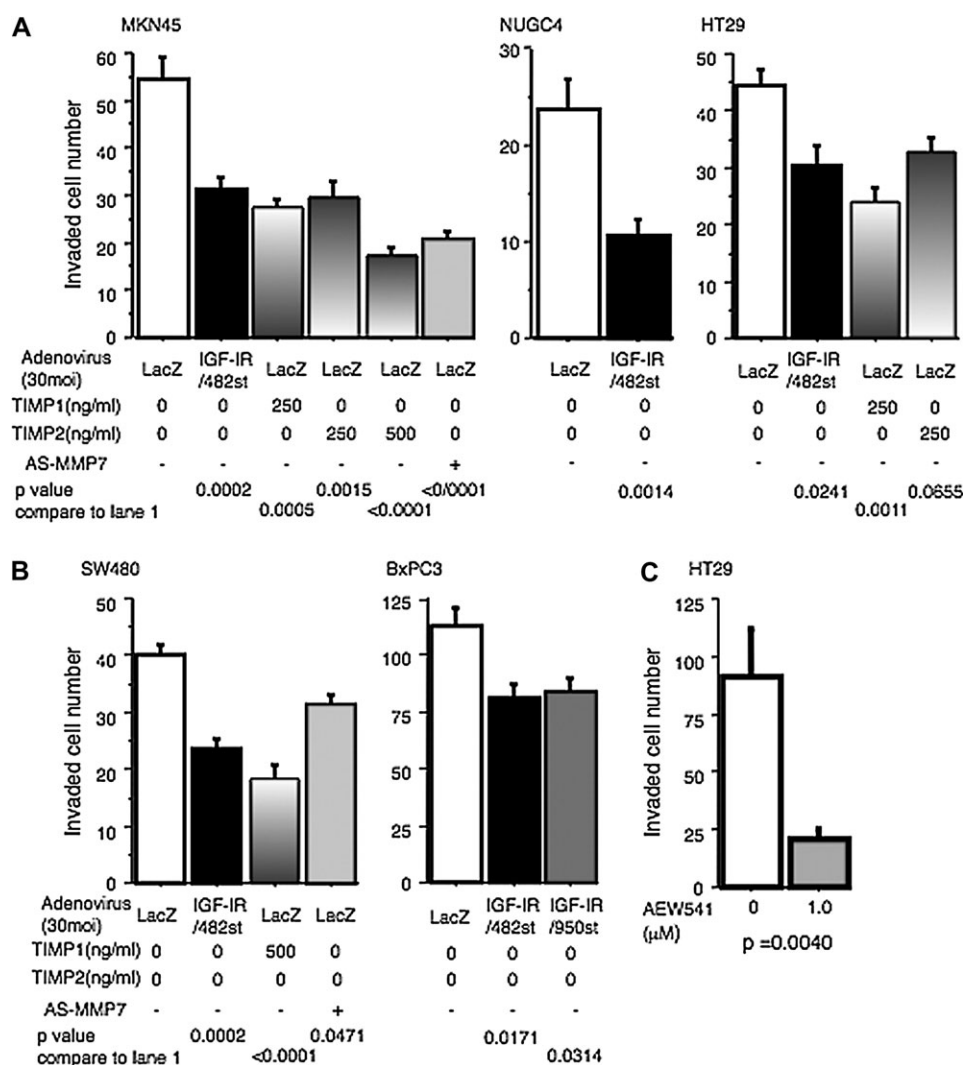


Fig. 4. Blockade of IGF-IR as well as inhibitions of MMPs reduce *in vitro* invasiveness of GI cancer cells. Invasion assays were performed triplicate and then analyzed. (A) The number of invaded MKN45 cell was reduced by either treatment with IGF-IR/dn, TIMP-1, TIMP-2 or antisense matrilysin (AS-MMP7). The effect of TIMP-2 showed dose dependency. Invasiveness of another gastric cancer cell line NUGC4 was also blocked by either IGF-IR/482st. In HT29 cells, the invasiveness was also blocked by either IGF-IR/dn or TIMPs. (B) In another colon cancer cell line SW480, either IGF-IR/dn, TIMP1 or antisense matrilysin reduced its invasive potential. Two different dominant negatives for IGF-IR inhibited invasion of BxPC-3. (C) A tyrosine kinase inhibitor, 1 μM AEW541, reduced the number of invaded HT29.

between 500 and 1000 mm³, 0/1 with TKI and 1/3 with control were invasive; in tumors >1000 mm³, 1/3 with TKI and 4/4 with control showed invasion. Immunohistochemical evaluation revealed that HT29 tumor treated with control vehicle expressed matrilysin; however, matrilysin expression in those treated with AEW541 was significantly reduced (Figure 5D). Immunohistochemical expression of phosphorylated Akt-1 was also downregulated in tumor treated with TKI; however, as seen *in vitro*, the expression of IGF-IR in HT29 tumor xenografts was not influenced by IGF-IR inhibition. The intensity of immunohistochemical staining in the positive areas was quantitatively measured using NIH image software and confirmed that AEW-541 inhibited both matrilysin expression and activation of Akt without influencing IGF-IR expression (Figure 5E).

Similarly, mice bearing established subcutaneous BxPC3 tumors were treated with NVP-AEW541 orally for seven successive days. Three days after treatment, mice were killed. Immunohistochemical staining showed that both expressions of matrilysin and phosphorylated Akt in tumor cells were reduced by AEW541, and the expression of IGF-IR was not changed (Figure 5F). These data support

our hypothesis that the effects of IGF-IR/dn on invasion and progression of GI tumor xenografts occur through blocking matrilysin expression.

Discussion

The object of these studies was to clarify the mechanisms by which IGF-IR/dn reduced the invasiveness of xenografted tumors in mice (36,37). We determined that blocking IGF-IR downregulates matrilysin expression in GI cancer cells and that this downregulation explained the reduction in invasion.

Recently, several relationships between IGF/IGF-IR axis and MMPs have been reported. Although IGF/IGF-IR axis is known to induce MMP-9 in breast cancer (40) and MMP-2 in lung cancer (41), this is a first report that IGF-IR signaling stimulates MMP expression in GI cancers. IGF-IR controls tumor cell invasion by coordinately regulating matrilysin expression and activating PI3-K/Akt-1 signaling, both critical to the behavior of GI cancer cells. These results are similar to the effects on MMP-2 expression previously observed in lung cancer cells (42).

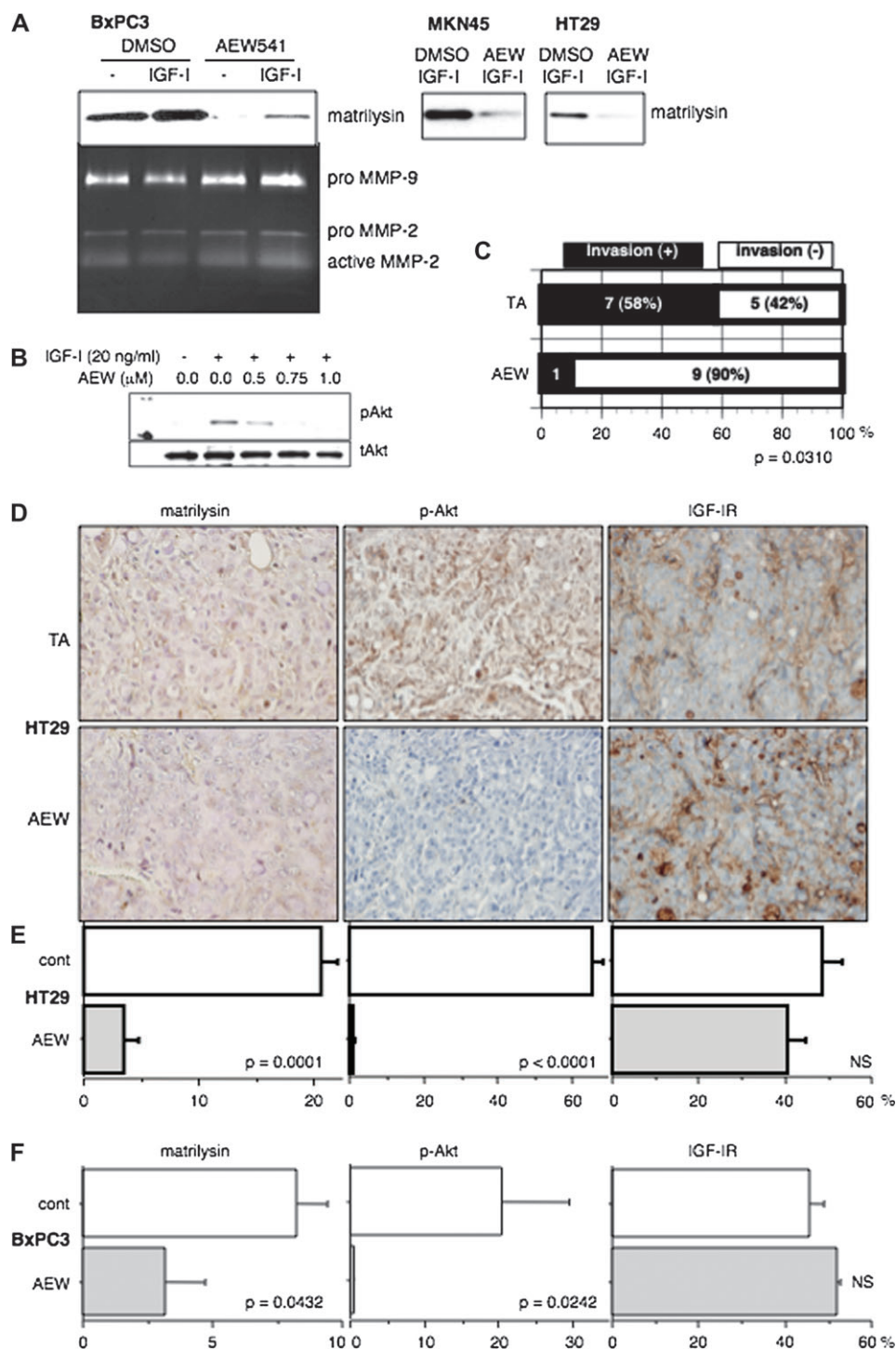


Fig. 5. NVP-AEW541 reduces expression of matrilysin and *in vivo* invasiveness. (A) In BxPC3 cells, 1 μ M AEW541 reduced 100 nM IGF-I upregulation of matrilysin expression analyzed by western blotting. Gelatin zymography shows that NVP-AEW541 did not affect the activities of MMP-2 and -9. Western blotting reveals that 1 μ M AEW541 reduced 100 ng/ml IGF-I-stimulated matrilysin expression in both MKN45 and HT29. Western blots and zymographies are representative of triplicate experiments. (B) Western blot analysis shows that 20 ng/ml IGF-I phosphorylated Akt-1 in BxPC3 and NVP-AEW541 blocked this phosphorylation in a dose-dependent fashion. (C) Oral administration of 40 mg/kg NVP-AEW541 reduced significantly invasive subcutaneous HT29 tumors on nude mice ($P = 0.0310$, Fisher's exact test). (D) NVP-AEW541 blocked both matrilysin expression and phosphorylation of Akt in HT29 tumor on nude mice, without affecting IGF-IR expression ($\times 400$). Immunohistochemical positive area was then quantitatively measured using NIH image software and confirmed the results on both HT29 (E) and BxPC3 (F) tumors on mice.

It has become apparent that the MMPs are exquisite regulators of cell-to-cell communication by virtue of their ability to process many non-matrix molecules, such as cytokines and growth factors. For example, matrilysin proteolysis of IGFBP3 plays a crucial role in upregulating IGF-I bioavailability in HT29 and COLO201 cells

(43). Matrilysin can cleave all six IGFBPs and can thus cause increased IGF-mediated IGF-1R phosphorylation (44). Moreover, matrilysin is also able to generate bioactive IGF-II by degrading the IGF-II/IGFBP-2 complex binding to heparan sulfate proteoglycan in the extra cellular matrix of HT29 (45). In addition, matrilysin can

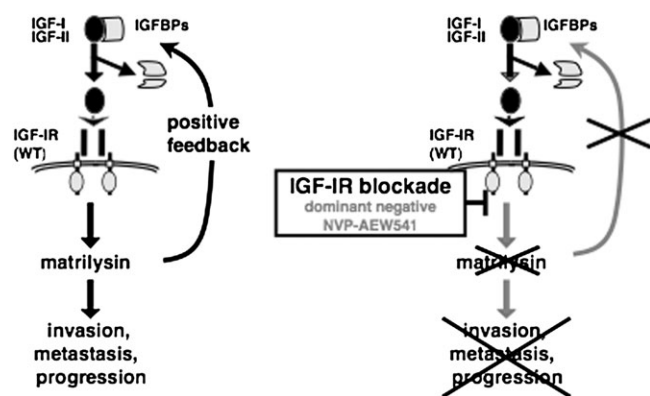


Fig. 6. IGF/IGF-IR and matrilysin positive feedback loop. IGF/IGF-IR axis promotes invasion, metastasis and progression of GI cancers via matrilysin. Matrilysin degrades IGF-BPs and releases free IGFs, which can then activate IGF-IR. Strategies that block IGF-IR prevent GI cancer progression through matrilysin downregulation.

activate pro-secreted form of a disintegrin and metalloproteinase 28 (ADAM28) that digests IGF-BP-3 and enhances the bioactivity of IGF-I (46,47). These data indicate that matrilysin may enhance both carcinogenesis and progression of IGF-IR-expressing tumors. In addition to these data, we have also shown that IGF/IGF-IR can upregulate matrilysin expression, indicating that there might be a positive feedback loop involving IGF-IR and matrilysin in tumor progression (Figure 6). Blocking this novel positive feedback loop may be important for the effective treatment of GI cancers.

The promoter regions of the matrilysin gene contain binding sites for both Ets and activator protein-1 transcriptional factors that mediate oncogene- and growth factor-induced transcription of many genes (21). IGF signaling is also known to stimulate these promoters (48). Thus, IGF-IR signals may stimulate activator protein-1 and/or Ets to upregulate matrilysin expression and these studies are planned.

In this study, we use both IGF-IR/dn and NVP-AEW541, which successfully reduced cancer cell invasion both *in vitro* and *in vivo*. We cannot exclude the possibility that both strategies reduce invasiveness through growth inhibition, however, that effect is greater than might be expected by the degree of growth inhibition, and the effects on MMPs are clear. Two other studies have reported the effectiveness of IGF-IR inhibitors in reducing MMP expression. One showed that the IGF-IR inhibitor picropodophyllin (PPP) decreased migration and invasion through downregulating MMP-2 expression in melanoma cells (49). Another is that (–)-epigallocatechin-3-gallate (ECGC) causes a decrease in the levels of messenger RNAs that encode MMPs-7 and -9 in colon cancer SW837 cells (50). Unfortunately, several MMP inhibitors have been tried but failed in clinical studies. In this study, both of the agents we used for targeting IGF-IR showed almost same effectiveness in blocking *in vitro* invasion as did direct inhibitors of matrilysin. Targeting IGF-IR may therefore be a strategy with both direct antitumor as well as antimetastatic/invasive activity in patients with GI cancers (Figure 6).

In this study, we have identified a novel positive feedback loop between the IGF/IGF-IR axis and matrilysin in the progression and invasiveness of GI cancers, and this link may be an important target of future therapeutic strategies for GI cancers.

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