

Hepatocyte growth factor-induced mesenchymal-epithelial transition factor activation leads to insulin-like growth factor 1 receptor inhibitor unresponsiveness in gastric cancer cells

RUJIAO LIU^{1,2*}, WENBO TANG^{1,2*}, XIAOTIAN HAN^{2,3}, RUIXUAN GENG⁴,
CHENCHEN WANG^{1,2} and ZHE ZHANG^{1,2}

¹Department of Medical Oncology, Fudan University Shanghai Cancer Center; ²Department of Oncology, Shanghai Medical College, Fudan University; ³Department of Gynecologic Oncology, Fudan University Shanghai Cancer Center, Shanghai 200032; ⁴International Medical Services, Peking Union Medical College Hospital, Beijing 100000, P.R. China

Received February 10, 2018; Accepted August 31, 2018

DOI: 10.3892/ol.2018.9414

Abstract. Insulin-like growth factor 1 receptor (IGF-1R) inhibitors have been developed as potential therapeutics for cancer treatment; however, the phase III trials have not produced promising overall survival rates. Therefore, understanding the mechanism underlying intrinsic resistance to IGF-1R-targeted agents is urgently required. A number of studies have revealed that activation of alternative receptor tyrosine kinases can mediate resistance to IGF-1R-targeted therapy. The present study investigated whether activated mesenchymal-epithelial transition factor (MET; also known as c-Met and hepatocyte growth factor receptor) confers resistance to an IGF-1R inhibitor (NVP-AEW541) of gastric cancer (GC) cells. NCI-N87 and MGC-803 cells were treated with varying concentrations and combinations of NVP-AEW541, hepatocyte growth factor (HGF) and MET small interfering (si)-RNA or crizotinib (a MET inhibitor). The effects of these agents on cell proliferation and pro-apoptotic events were assessed by Cell Counting Kit-8 assays and flow cytometry. Receptor activation and the downstream signaling pathway were examined using western blot analysis. Expression and/or activation of MET and IGF-1R in 156 GC specimens were evaluated

by immunohistochemistry. The results demonstrated that NVP-AEW541 inhibited cell growth, with dephosphorylation of IGF-1R and protein kinase B (AKT), in NCI-N87 and MGC-803 cells. Application of HGF activated MET and the downstream AKT signaling pathways, decreased apoptotic events and restored cell proliferation, which were reversed by MET inhibition via crizotinib or siRNA knockdown. Furthermore, combination therapy of NVP-AEW541 and crizotinib exhibited an enhanced effectiveness *in vitro*. In addition, >40% of IGF-1R overexpressed GC specimens showed MET expression and activation. In conclusion, HGF-induced MET activation may represent a novel mechanism conferring unresponsiveness to IGF-1R-targeted agents in GC, and inhibition of MET may improve the efficacy of IGF-1R inhibitors.

Introduction

Gastric cancer (GC) is the fourth most common type of malignant tumor and is the second leading cause of cancer-associated mortality (1). Recent therapeutic strategies have focused on molecularly targeted therapies, evaluating the numerous successes of molecular targeted therapy in different types of cancer, as well as the absence of overlapping toxicity with current cytotoxic drugs.

Insulin-like growth factor 1 receptor (IGF-1R) has been proposed as a potentially effective target for cancer treatment, as it serves an important role in tumor cell survival and tumorigenesis via the mitogen-activated protein kinase (MAPK) and protein kinase B (AKT) signaling pathways; its overexpression has been reported in many types of cancer (2-4). Early phase clinical trials of the IGF-1R monoclonal antibody (mAb) figitumumab revealed anticancer activities in non-small-cell lung cancer with an improved objective response rate (from 42 to 54%) (5,6). Unfortunately, phase III trials of figitumumab were recently discontinued as the interim analysis indicated that the IGF-1R mAb was unlikely to improve overall survival. Thus, understanding of the underlying mechanisms of intrinsic resistance to IGF-1R-targeted therapies is urgently required.

Correspondence to: Dr Zhe Zhang, Department of Medical Oncology, Fudan University Shanghai Cancer Center, 270 Dong'an Road, Xuhui, Shanghai 200032, P.R. China
E-mail: zhangzhe2010fuduscc@gmail.com

*Contributed equally

Abbreviations: PI3K, phosphoinositide 3-kinase; MAPK, mitogen-activated protein kinase; ALK, anaplastic lymphoma kinase; RPMI, Roswell Park Memorial Institute medium; DMSO, dimethyl sulfoxide; RIPA, radioimmunoprecipitation assay

Key words: insulin-like growth factor 1 receptor inhibitor, mesenchymal-epithelial transition factor, hepatocyte growth factor, drug resistance, gastric cancer

Mesenchymal-epithelial transition factor (MET), also known as c-Met, the receptor tyrosine kinase (RTK) for hepatocyte growth factor (HGF), is frequently amplified and/or overexpressed in GC (7,8). MET activation through amplification or HGF leads to a cascade of events, including MAPK and AKT signaling, common downstream targets of the IGF-1R. An increasing body of evidence has supported that 'cross-talk' between RTKs may result in the activation of one such receptor via signaling pathways mediated by a different RTK. In addition, activation of bypass RTKs is now considered to be a widespread innate or acquired resistance mechanism in targeted therapies (9-12). Previous studies have revealed that activation of epidermal growth factor receptor and insulin receptor (IR) may represent potential resistance mechanisms to IGF-1R-targeted therapy via alternative signaling pathways (13,14). Cross-talk between IGF-1R and MET has been reported previously, as in cancer cells co-expressing IGF-1R and MET, IGF-1R activation lead to a delayed phosphorylation of MET, independent of HGF (15); MET is also required for IGF-I-mediated migration and invasion (16,17).

Therefore, the aim of the present study was to determine whether MET activation had an impact on resistance to the IGF-1R tyrosine kinase inhibitor (TKI), NVP-AEW541, in GC cell lines. It was observed that HGF-induced MET activation led to resistance to IGF-1R TKI by restoring AKT pathway signaling, which was reversed by crizotinib application, a MET TKI (18), or MET silencing. Furthermore, MET activation was observed more frequently in GC patients with IGF-1R overexpression. Understanding the cross-talk between IGF-1R and MET could be helpful for patient selection and treatment strategies for IGF-1R inhibitors.

Materials and methods

Cell lines. NCI-N87 and HGC-27 human GC cells were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). MKN-45, MKN-28 and MGC-803 GC cells were obtained from 3D Biopharm Biotech Co., Ltd. SNU-216 cells were sourced from Medical College of Xiamen University (Fuzhou, China). Cell lines were tested and authenticated by short tandem repeat DNA profiling analysis. Particularly, MKN28 cell line has been reported to be contaminated, the contaminating cell line is MKN74, of the gastric tubular adenocarcinoma type (19). Cells were cultured in minimum essential medium (for HGC-27 cells) or in RPMI-1640 medium containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin-streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere with 5% carbon dioxide.

Chemicals. NVP-AEW541 was provided by Novartis International AG (Basel, Switzerland) and crizotinib was purchased from Selleck Chemicals (Houston, TX, USA). Recombinant human HGF was purchased from PeproTech, Inc., (Rocky Hill, NJ, USA). Compounds were dissolved in 100% dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and diluted with culture medium to the desired concentration with a final DMSO concentration of <0.2% (v/v). DMSO was added to culture medium as a solvent control.

Cell viability assays. Assessment of cell viability was performed as follows. Cells were seeded at 5,000 cells per well in 96-well plates and incubated for 24 h. On the following day, the cells were treated with increasing concentrations of the indicated drugs for 72 h. Treatment with each concentration was performed in 6 replicate wells and repeated at least 3 times. Cell viability was determined using Cell Counting Kit (CCK)-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's instructions. Median inhibition concentration values were calculated using a nonlinear regression model and GraphPad Prism v.5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Clonogenic assays and growth curves. Briefly, NCI-N87 cells were seeded at 4×10^4 cells per well and MGC-803 cells at 1×10^4 cells per well in 12-well plates, and cultured in the absence and presence of the indicated concentrations of drug alone or in a combination of the drugs or cytokines as required for 2 weeks. Following fixation of the cells with 4% paraformaldehyde, cell numbers were quantified by staining the cells with 0.1% crystal violet (Sigma-Aldrich; Merck KGaA), extracting dye with 10% acetic acid and then the optical density was determined at 600 nm. Multiple growth curves were produced in triplicate experiments.

RNA interference transfection. For small interfering (si)-RNA experiments, N87 or MGC-803 cells were firstly seeded, then following 24 h, MET siRNA or scrambled siRNA (sense 5'-CAACACCCATCCAGAATGTCA-3' and antisense 5'-TGACATTCTGGATGGGTGTTG-3'; final concentration, 10 nmol/l) was incubated with HiperFect Transfection Reagent to allow for the formation of transfection complexes, which were then added to cells for transfection. Scrambled siRNA (Qiagen, Inc., Valencia, CA, USA) was used as the negative control. Following 24 h post-transfection, the medium was removed and cells were treated as indicated. Cell viability was determined using a CCK-8 Cell Viability Assay or Clonogenic Assay (performed as aforementioned).

Western blot analysis. Cells were lysed in radioimmunoprecipitation assay buffer supplemented with a complete protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). Protein content in the cell lysates was quantified using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China) to ensure that all samples contained similar amounts of protein. Antibodies against MET (25H2), phosphorylated (p)-MET (Tyr1234/1235), IGF-1R β , p-IGF-1R β (Tyr1135/1136), AKT, p-Akt (Ser473), p42/44 MAP, and p-p42/44 MAPK (Thr202/Tyr204) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies against β -actin were from Jackson Laboratory (Bar Harbor, ME, USA). Blots were probed with the indicated primary antibodies, then with horseradish peroxidase (HRP)-conjugated secondary antibody. Proteins were detected using the enhanced chemiluminescence reagent, Pierce Enhanced Chemiluminescence Western Blotting Substrate (Thermo Fisher Scientific, Inc.).

Detection of apoptosis. Cell apoptosis was detected using an Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to

the manufacturer's protocol. Briefly, NCI-N87 and MGC-803 cells (6×10^5 per well) were cultured in 6-well plates and treated with either NVP-AEW541 or HGF alone or in combination with crizotinib for 12 h. The floating and trypsinized adherent cells were then harvested and stained with Annexin V and propidium iodide. Quantitative analysis was performed with a Cytomics FC 500 flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) using CXP software (Beckman Coulter, Inc.). Cells positive for early apoptosis exhibited Annexin V-FITC staining only. Assays were performed in triplicate.

Patients and specimens. A total of 156 primary gastric adenocarcinoma specimens were obtained by surgical resection between 2007 and 2010 at Shanghai Cancer Hospital, Fudan University (Shanghai, China). Samples were acquired with patient written informed consent, using the protocol approved by the Shanghai Cancer Hospital Research Ethics Committee. Paraffin blocks were selected according to the availability of suitable formalin-fixed, paraffin-embedded tissue and complete clinicopathologic and follow-up data ($n=154$ samples). Tumor staging was determined according to the Tumor-Node-Metastasis classification system of the American Joint Committee on Cancer, 7th edition.

Tissue microarray and immunohistochemistry (IHC). Tissue microarrays were constructed in collaboration with Shanghai Biochip (Shanghai, China), as described previously (20). All samples from GC patients were reviewed histologically following hematoxylin and eosin staining. Representative cores were taken from paraffin blocks, in selected areas away from necrotic and hemorrhagic regions.

The commercially available primary antibodies used for IHC assays were as follows: Antibodies against IGF-1R and p-IGF-1R (Y1161) were from Abcam (Cambridge, UK). An anti-Total c-MET (SP44) rabbit monoclonal primary antibody was from Roche Diagnostics. Antibodies against p-MET (Tyr1234/1235) were from Cell Signaling Technology, Inc. IHC of paraffin sections was performed using a 2-step protocol (MaxVision HRP-Polymer Detection System; Fuzhou Maixin Biotech Co., Ltd., Fuzhou, China) according to the manufacturer's instructions. Briefly, paraffin sections were deparaffinized and then rehydrated. Following microwave antigen retrieval, endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide. Non-specific binding sites were blocked with phosphate buffered saline containing 10% normal goat serum. Following staining with primary antibodies and peroxidase polymer-conjugated secondary antibody, sections were incubated with diaminobenzidine solution and counterstained with hematoxylin. Negative control slides without the primary antibodies were included for all samples.

Immunostaining scoring system. Slides were independently evaluated by two investigators who were blinded to the clinical information. For MET and IGF-1R staining, only tumor cell membrane staining was considered (2,21,22). Immunostaining was scored according to a semiquantitative 4-grade scale, as follows: 0, no positive staining or $<10\%$ positively stained cells in tumor; 1+, 10–40% positively stained cells in tumor; 2+, 40–70% positively stained cells in tumor; and 3+, $>70\%$ positively

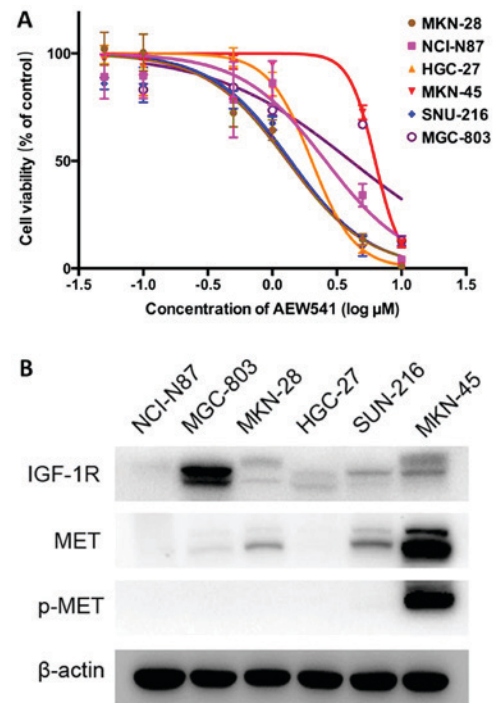


Figure 1. MET-overexpressed GC cell line displays the most unresponsive-ness to NVP-AEW541. (A) Six GC cell lines were treated with increasing concentrations of NVP-AEW541 for 72 h, followed by evaluation of cell proliferation and the IC_{50} . Data points indicate the average of 6 replicates and are presented as the mean \pm standard error of the mean. (B) Protein lysates were collected from all 6 GC cell lines and analyzed for baseline IGF-1R and MET expression and activation. β -actin served as a loading control. MET, mesenchymal-epithelial transition; IC_{50} , half-maximal inhibitory concentration; IGF-1R, insulin-like growth factor 1 receptor. GC, gastric cancer.

stained cells in tumor. Samples assigned scores of 2+ or 3+ were considered to have overexpression of the respective protein, and those with scores of 0 or 1+ were considered to have no expression or moderate expression. p-MET and p-IGF-1R were scored as follows: 0, absent; 1+, weak; 2+, moderate; and 3+, strong, with consideration of the proportion and intensity of the staining pattern in tumor cytoplasm, membrane or nucleus (21).

Statistical analysis. Statistical analyses were performed with GraphPad Prism v.7.0 (GraphPad Software, Inc., La Jolla, CA, USA). The data of functional experiments were expressed as the mean \pm standard deviation from three independent experiments. IC_{50} of the GC cells were calculated using a nonlinear regression model. Differences between cell viability and growth curves were analyzed by two-way ANOVA followed by the Bonferroni multiple comparison test. Quantitative variables were analyzed by the Student's t-test or one-way ANOVA with Bonferroni posttest. All tests were two-sided. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

MET expression impacts NVP-AEW541 sensitivity. The GC cell lines selected for the present study, MKN-28, SNU-216, NCI-N87, MGC-803, HGC-27 and MKN-45, displayed varying sensitivities to IGF-1R inhibition by NVP-AEW541 (Fig. 1A). As shown in Fig. 1B, expression of IGF-1R was not necessarily the only factor able to determine sensitivity to NVP-AEW541.

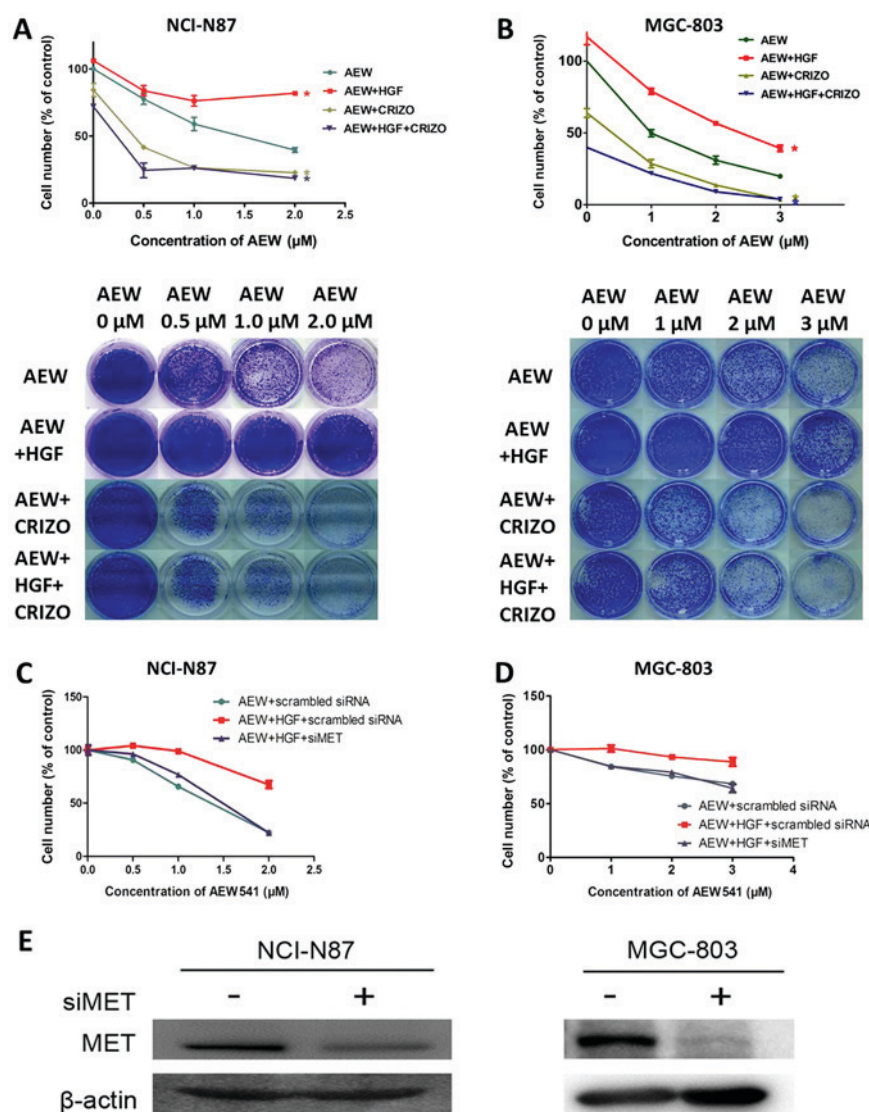


Figure 2. NVP-AEW541 sensitivity is abrogated by treatment with HGF in NCI-N87 and MGC-803 cells, which can be restored by MET blocking or silencing in the presence of HGF. (A) NCI-N87 cells were treated with NVP-AEW541 (AEW), NVP-AEW541+HGF 20 ng/ml, NVP-AEW541 + crizotinib 0.1 μ M (CRIZO) and NVP-AEW541+HGF+crizotinib 0.1 μ M, followed by determination of cell proliferation at 2 weeks. (B) MGC-803 cells were treated with NVP-AEW541 (AEW), NVP-AEW541+HGF 50 ng/ml, NVP-AEW541 + crizotinib 0.5 μ M (CRIZO) and NVP-AEW541+HGF+crizotinib 0.5 μ M, followed by determination of cell proliferation at 2 weeks. Data points indicate the average of 3 replicates and are presented as the mean \pm standard error of the mean. * P <0.001 vs. NVP-AEW541-treated control group. (C) NCI-N87 cells were treated with NVP-AEW541 (AEW)+scrambled siRNA, AEW+HGF (20 ng/ml) +scrambled siRNA and AEW+HGF+MET siRNA, followed by determination of cell proliferation at 1 week. Data points indicate the average of 3 replicates and are presented as the mean \pm standard error of the mean. (D) MGC-803 cells were treated with NVP-AEW541 (AEW)+scrambled siRNA, AEW+HGF (50 ng/ml) +scrambled siRNA, and AEW+HGF+MET siRNA, followed by determination of cell proliferation at 1 week. Data points indicate the average of 3 replicates of three and are presented as the mean \pm standard error of the mean. (E) NCI-N87 and MGC-803 cells were transfected with MET or scrambled siRNA. The indicated gene expression was measured by western blotting following 3 days of 20 nM siRNA transfection. MET was significantly knocked down by the indicated siRNA. MET, mesenchymal-epithelial transition; HGF, hepatocyte growth factor; siRNA, small interfering RNA.

Notably, MKN-45, which had significantly enhanced expression and activation of MET, exhibited the greatest levels of unresponsiveness to NVP-AEW541. Accordingly, it was hypothesized that MET expression may have an impact on NVP-AEW541 sensitivity. Particularly, although MKN28 cell line is of mixed GC type, this has no impact on interpretation of this figure. Accordingly, it was hypothesized that MET expression may have an impact on NVP-AEW541 sensitivity.

NVP-AEW541 sensitivity is abrogated by HGF treatment in NCI-N87 and MGC-803 cells. When NCI-N87 and MGC-803 cells were exposed to NVP-AEW541, NVP-AEW541 exerted a cancer cell inhibitory effect in a dose-dependent manner. To

verify the role of MET activation in conferring NVP-AEW541 resistance, NCI-N87 and MGC-803 cells were treated with HGF, the corresponding MET ligand, and then exposed to NVP-AEW541. HGF significantly increased the number of NVP-AEW541-resistant colonies in NCI-N87 and MGC-803 GC cells (P <0.001), thereby attenuating the inhibitory effect of NVP-AEW541 (Fig. 2).

NVP-AEW541 sensitivity is restored by MET blocking or silencing in the presence of HGF. The MET TKI crizotinib alone caused moderate cell growth inhibition only. When NCI-N87 and MGC-803 cells were treated with a combination of NVP-AEW541, HGF and crizotinib, HGF-mediated resistance to

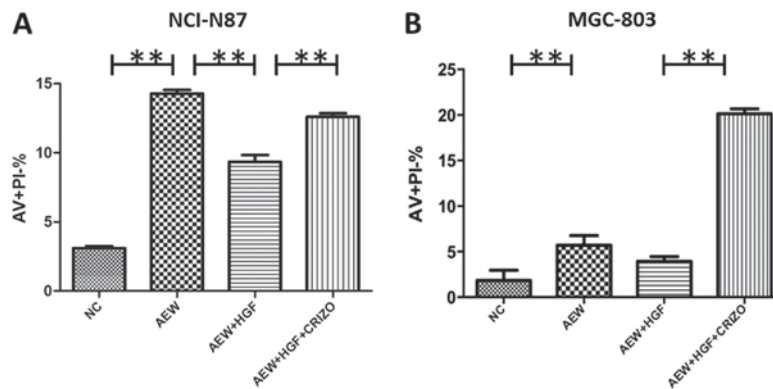


Figure 3. HGF decreases the number of apoptotic events in NVP-AEW541-treated NCI-N87 and MGC-803 cells. (A) NCI-N87 cells were treated for 18 h with media, NVP-AEW541 15 μ M (AEW), NVP-AEW541+HGF 50 ng/ml and NVP-AEW541+HGF+crizotinib 2 μ M (CRIZO), and then analyzed by flow cytometry. (B) MGC-803 cells were treated for 18 h with media, NVP-AEW541 20 μ M (AEW), NVP-AEW541+HGF 100 ng/ml and NVP-AEW541+HGF+crizotinib 10 μ M (CRIZO), and then analyzed by flow cytometry. ** P <0.05. The results are the average of triplicate experiments and data are presented as the mean \pm standard deviation. HGF, hepatocyte growth factor.

NVP-AEW541 was abrogated completely and growth inhibitory effects were restored (Fig. 2A and B), which could be explained by MET blocking as opposed to the side effects of crizotinib.

As crizotinib is a multi-target inhibitor of RTKs, including anaplastic lymphoma kinase, MET, Recepteur d'Origine Nantais and ROS proto-oncogene 1 RTK (18), in order to validate that IGF-1R inhibitor resistance could be attenuated by MET inhibition, the present study knocked down the MET gene using specific siRNAs. Knockdown of MET was confirmed by western blot analysis in NCI-N87 and MGC-803 cells (Fig. 2E). When the two silenced groups in NCI-N87 and MGC-803 cells were treated with NVP-AEW541 and HGF, HGF-induced resistance to NVP-AEW541 was inhibited (Fig. 2C and D). Thus, it was indicated that HGF induced NVP-AEW541 resistance via MET activation.

HGF decreases the number of apoptotic events in NVP-AEW541-treated NCI-N87 and MGC-803 cells. NCI-N87 and MGC-803 cells underwent apoptosis when incubated for 18 h with NVP-AEW541. The results revealed that HGF reduced the percentage of pro-apoptotic cells in the NCI-N87 and MGC-803 cell lines from 14.3 to 9.3% (P <0.05) and 5.7 to 3.9%, respectively. Treating NCI-N87 and MGC-803 cells with a combination of HGF and crizotinib restored the pro-apoptotic effects of NVP-AEW541 by increasing the percentage of pro-apoptotic cells to 12.6 and 20.1%, respectively (P <0.05; Fig. 3).

HGF decreases NVP-AEW541 sensitivity via MET-dependent upregulation of AKT signaling. To explore the mechanism by which HGF decreased NVP-AEW541 sensitivity and the number of apoptotic events, the present study examined the activation status of downstream signaling molecules. MGC-803 and NCI-N87 cells were exposed to NVP-AEW541, HGF and crizotinib as indicated, and the activation status of membranous receptors and downstream signaling pathways were detected by western blotting (Fig. 4). Treatment with NVP-AEW541 resulted in the dephosphorylation of IGF-1R and AKT, as opposed to extracellular signal-regulated kinase, in NCI-N87 and MGC-803 cells. The presence of HGF led to MET phosphorylation and reactivation of AKT. When cells were treated with a combination of NVP-AEW541

and crizotinib, the inhibition of IGF-1R and MET led to the sustained inhibition of the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway, even in the presence of HGF, which suggested that dual receptor inhibition may lead to an optimal clinical efficacy.

Combined administration of NVP-AEW541 and crizotinib exerts an enhanced effectiveness. To evaluate the potential of combination therapy, the present study treated NCI-N87 and MGC-803 cells with NVP-AEW541 and crizotinib, and then analyzed the effects of dual receptor inhibition on cell viability. Treatment of NCI-N87 cells with either NVP-AEW541 or crizotinib resulted in a relatively modest inhibition response, whereas treatment with the drugs combined led to a significantly enhanced growth inhibition response (Fig. 5). In addition, MGC-803 cells exhibited a similar response to dual receptor inhibition, suggesting that combination therapy targeting IGF-1R and MET may be effective in the clinic.

MET expression and activation in GC samples overexpressing IGF-1R. To establish the potential of MET activation in IGF-1R inhibitor candidate biomarkers, the present study detected the expression and/or activation profile of MET and IGF-1R in 156 primary gastric surgical samples by IHC, and summarized the clinicopathologic characteristics of the patients (data not shown). A total of 154 primary GC samples were suitable for IHC assessment. MET, p-MET, IGF-1R and p-IGF-1R expression was detected in 68 (44.2%), 51 (33.1%), 120 (77.9%) and 86 tumors (55.8%), respectively. In addition, there were significantly more MET expressed and activated cases in patients with IGF-1R overexpressing tumors than in those with IGF-1R negative/moderately expressed tumors (MET, 69.8 vs. 26.4%, P <0.0001; p-MET, 42.9 vs. 26.4%, P <0.05; Table I). Two representative samples of patients with positive expression or activation of MET and IGF-1R are presented in Fig. 6.

Discussion

The role of IGF-1R in tumorigenesis and oncogenic transformation is well established as a cancer treatment target of

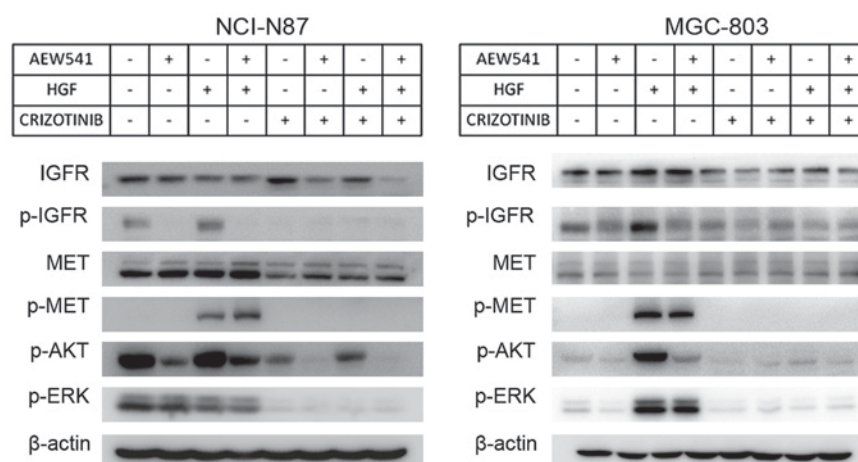


Figure 4. HGF-dependent phosphorylation of MET reactivates AKT, which is attenuated by crizotinib via MET inhibition. NCI-N87 cells were treated for 3 h with NVP-AEW541 15 μ M, and/or crizotinib 20 μ M with or without the addition of HGF 50 ng/ml for the final 30 min. MGC-803 cells were treated for 3 h with NVP-AEW541 15 μ M, IGF-1 50 ng/ml and/or crizotinib 10 μ M with or without the addition of HGF 10 ng/ml for the final 30 min. Whole cell lysates were analyzed by SDS-PAGE, followed by western blot analysis. HGF-induced MET phosphorylation restored AKT signaling in NVP-AEW541-treated cells; a combination of NVP-AEW541 and crizotinib treatment blocked the activation of AKT and MAPK in HGF-stimulated NCI-N87 cells, and AKT in HGF-stimulated MGC-803 cells. HGF, hepatocyte growth factor; MET, mesenchymal-epithelial transition; AKT, protein kinase B; IGF-1, insulin-like growth factor 1; MAPK, mitogen-activated protein kinase.

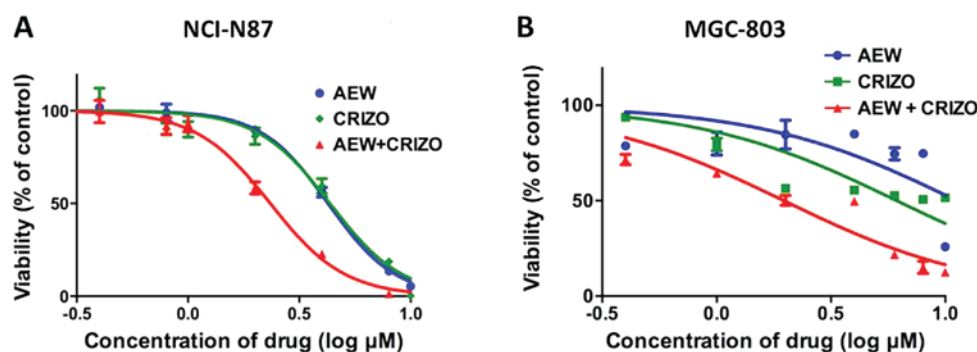


Figure 5. Combination of NVP-AEW541 (AEW) with crizotinib (CRIZO) results in a significant increase in cell growth toxicity. (A) NCI-N87 and (B) MGC-803 cells were treated with increasing concentrations of the indicated drugs for 72 h, followed by evaluation of cell viability by Cell Counting Kit-8. Data points indicate the average of 6 replicates and is presented as the mean \pm standard error of the mean.

particular interest. However, several phase III clinical trials have revealed that the IGF-1R inhibitor is less likely to be effective, even in cases expressing IGF-1R; thus, further investigation in the mechanisms underlying resistance are required. In the present study, the results demonstrated that MET activation is a novel mechanism of IGF-1R inhibitor resistance in GC cells by activating downstream AKT signaling and blocking MET via TKI or siRNA, thus, re-sensitizing GC cells to IGF-1R inhibition *in vitro*.

Inhibition of RTKs often results in the alternative activation of other RTKs, thus restoring the downstream PI3K and MAPK signaling pathways. Previous studies have revealed that insulin receptor activation represents a resistance mechanism that limits the efficacy of IGF-1R targeting (14,23); however, this cannot fully explain the results that the dual IGF-1R/IR inhibitor OSI-906 showed a lack of efficacy in investigational colorectal cancer patients.

The present study revealed that MET-activated MKN-45 cells, which have a similar IGF-1R expression profile to those of the IGF-1R inhibitor-sensitive HGC-27 and NCI-N87 cells, were resistant to the IGF-1R inhibitor, NVP-AEW541. This

result suggested that MET expression may have impact on NVP-AEW541 sensitivity.

HGF-induced MET activation can rescue NCI-N87 and MGC-803 cells from NVP-AEW541 mediated growth inhibition. MET phosphorylation reactivated the common downstream PI3K/AKT signaling pathway, subsequently decreasing the frequency of pro-apoptotic events in NCI-N87 and MGC-803 cells. Furthermore, administration of MET-TKI or MET knockdown reversed this effect and restored NVP-AEW541 sensitivity in HGF-treated cancer cells. In addition, crizotinib induced a prominent reduction in the level of AKT activation together with the IGF-1R inhibitor in the presence of HGF, resulting in a greater number of apoptotic events.

As the results revealed, treatment with NVP-AEW541 inhibited the cell growth of NCI-N87 and MGC-803 cells by blocking PI3K/AKT signaling as opposed to MAPK signaling; this result is in agreement with a previous study in which NVP-AEW541 had no or only a weak effect on inhibiting MAPK activation in esophageal, colon and biliary tract cancer (24-27). Knockdown of IGF-1R

Table I. Association between MET and IGF-1R expression status.

	IGF-1R IHC status			
IHC status	0 and 1 (%)	2 and 3 (%)	Total no. of patients	P-value
MET				
0	67 (73.6)	19 (30.2)	86 (55.8)	<0.0001
1, 2, and 3	24 (26.4)	44 (69.8)	68 (44.2)	
p-MET				
0	67 (73.6)	36 (57.1)	103 (66.9)	<0.05
1, 2, and 3	24 (26.4)	27 (42.9)	51 (33.1)	

MET, mesenchymal-epithelial transition factor; p, phosphorylated; IHC, immunohistochemistry; IGF-1R, insulin-like growth factor 1 receptor.

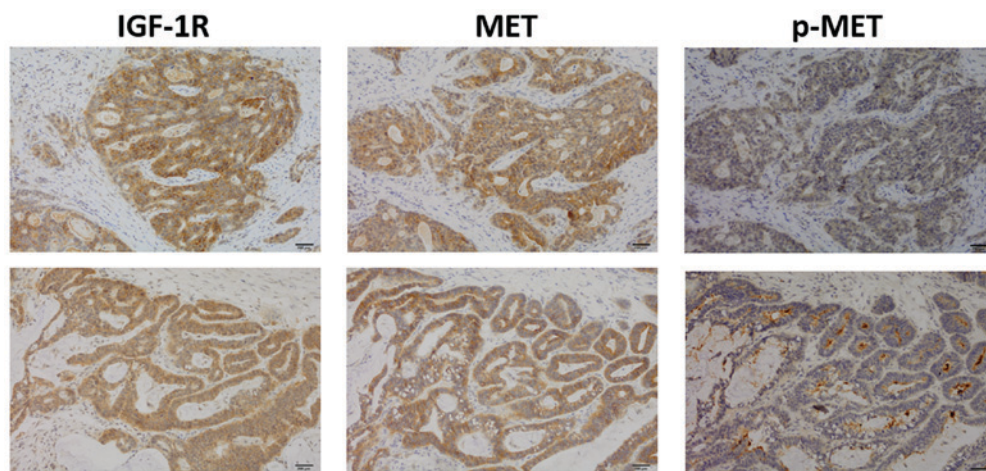


Figure 6. Immunohistochemical detection of IGF-1R, MET and p-MET in GC specimens. Typical examples of immunohistochemical staining for IGF-1R, MET and p-MET. Magnification, x200. GC, gastric cancer; IGF-1, insulin-like growth factor 1 receptor; MET, mesenchymal-epithelial transition; p-, phosphorylated.

inhibited the phosphorylation of AKT; however, it had no effect on ERK (28). This suggested that the bioactivity of the IGF-1/IGF-1R axis may be more dependent on the PI3K/AKT signaling pathway compared with the MAPK signaling pathway; however, on the other hand, sustained MAPK activity may have a potential influence on limiting the efficacy of the IGF-1R inhibitor.

Additionally, as shown in the clonogenic assay, crizotinib had little effect as a single agent on NCI-N87 and MGC-803 cell proliferation, indicating that crizotinib alone did not exhibit marked antitumor activity. It was also noted that there was dephosphorylation of IGF-1R in NCI-N87 and MGC-803 cells treated with crizotinib. This result is consistent with several studies supporting cross activation between IGF-1R and MET receptors (16,29). It is likely that blocking one of the two receptors will lead to reshuffling of the downstream signaling pathways and in turn, affect the other receptor (30).

Notably, co-expression of MET and IGF-1R is a common phenomenon and has been detected in a broad range of types of cancer (18,31,32). Although MET gene amplification accounts for a small subset of GCs (7,33,34), high levels of MET protein and MET activation are commonly encountered (7). In the present study, MET expression and activation were observed

in 44.2 and 33.1% gastric tumor samples, respectively. Furthermore, a large proportion of IGF-1R overexpressing gastric tumors (42.9%) have MET activation, which may lead to IGF-1R/IR inhibitor resistance. This result established the feasibility of patient enrichment with a MET profile, and the potential clinical application of combined therapy.

In conclusion, to the best of our knowledge, this is the first study to show that HGF-induced MET activation represents a novel resistance mechanism of IGF-1R inhibition in GC cells. Therefore, the detection of MET receptor status should be taken into consideration when recruiting patients into IGF-1R inhibitor clinical trials, and simultaneous MET inhibition may be required to overcome primary and/or acquired IGF-1R inhibitor resistance.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Natural Science Foundation of Shanghai (grant no. 15ZR1407900) and the Natural Science Foundation of China (grant no. 81772574).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

RL and WT performed the majority of the experiments and drafted the manuscript. XH and RG performed the experiments. CW collected the tumor samples used in this study. ZZ conceived the project and supervised the research. All authors participated in the design and coordination of the study, and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from all patients. The study was approved by the Institutional Review Board of the Fudan University Shanghai Cancer Center.

Patient consent for publication

Written informed consent was obtained from all patients.

Competing interests

The authors declare that they have no competing interests.

References

- Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J and Jemal A: Global cancer statistics, 2012. *CA Cancer J Clin* 65: 87-108, 2015.
- Matsubara J, Yamada Y, Hirashima Y, Takahari D, Okita NT, Kato K, Hamaguchi T, Shirao K, Shimada Y and Shimoda T: Impact of insulin-like growth factor type 1 receptor, epidermal growth factor receptor and HER2 expressions on outcomes of patients with gastric cancer. *Clin Cancer Res* 14: 3022-3029, 2008.
- Pollak M: The insulin and insulin-like growth factor receptor family in neoplasia: An update. *Nat Rev Cancer* 12: 159-169, 2012.
- Samani AA, Yakar S, LeRoith D and Brodt P: The role of the IGF system in cancer growth and metastasis: Overview and recent insights. *Endocr Rev* 28: 20-47, 2007.
- Karp DD, Paz-Ares LG, Novello S, Haluska P, Garland L, Cardenal F, Blakely LJ, Eisenberg PD, Langer CJ, Blumenschein G Jr, *et al*: Phase II study of the anti-insulin-like growth factor type 1 receptor antibody CP-751,871 in combination with paclitaxel and carboplatin in previously untreated, locally advanced, or metastatic non-small-cell lung cancer. *J Clin Oncol* 27: 2516-2522, 2009.
- Gualberto A and Pollak M: Emerging role of insulin-like growth factor receptor inhibitors in oncology: Early clinical trial results and future directions. *Oncogene* 28: 3009-3021, 2009.
- Janjigian YY, Tang LH, Coit DG, Kelsen DP, Francone TD, Weiser MR, Jhanwar SC and Shah MA: MET expression and amplification in patients with localized gastric cancer. *Cancer Epidemiol Biomarkers Prev* 20: 1021-1027, 2011.
- Inoue T, Kataoka H, Goto K, Nagaike K, Igami K, Naka D, Kitamura N and Miyazawa K: Activation of c-Met (hepatocyte growth factor receptor) in human gastric cancer tissue. *Cancer Sci* 95: 803-808, 2004.
- Wilson TR, Fridlyand J, Yan Y, Penuel E, Burton L, Chan E, Peng J, Lin E, Wang Y, Sosman J, *et al*: Widespread potential for growth-factor-driven resistance to anticancer kinase inhibitors. *Nature* 487: 505-509, 2012.
- Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, Lindeman N, Gale CM, Zhao X, Christensen J, *et al*: MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science* 316: 1039-1043, 2007.
- Shattuck DL, Miller JK, Carraway KL III and Sweeney C: Met receptor contributes to trastuzumab resistance of Her2-overexpressing breast cancer cells. *Cancer Res* 68: 1471-1477, 2008.
- Yano S, Wang W, Li Q, Matsumoto K, Sakurama H, Nakamura T, Ogino H, Kakiuchi S, Hanibuchi M, Nishioka Y, *et al*: Hepatocyte growth factor induces gefitinib resistance of lung adenocarcinoma with epidermal growth factor receptor-activating mutations. *Cancer Res* 68: 9479-9487, 2008.
- Huang F, Greer A, Hurlburt W, Han X, Hafezi R, Wittenberg GM, Reeves K, Chen J, Robinson D, Li A, *et al*: The mechanisms of differential sensitivity to an insulin-like growth factor-1 receptor inhibitor (BMS-536924) and rationale for combining with EGFR/HER2 inhibitors. *Cancer Res* 69: 161-170, 2009.
- Buck E, Gokhale PC, Koujak S, Brown E, Eyzaguirre A, Tao N, Rosenfeld-Franklin M, Lerner L, Chiu MI, Wild R, *et al*: Compensatory insulin receptor (IR) activation on inhibition of insulin-like growth factor-1 receptor (IGF-1R): Rationale for cotargeting IGF-1R and IR in cancer. *Mol Cancer Ther* 9: 2652-2664, 2010.
- Varkaris A, Gaur S, Parikh NU, Song JH, Dayyani F, Jin JK, Logothetis CJ and Gallick GE: Ligand-independent activation of MET through IGF-1/IGF-1R signaling. *Int J Cancer* 133: 1536-1546, 2013.
- Bauer TW, Somcio RJ, Fan F, Liu W, Johnson M, Lesslie DP, Evans DB, Gallick GE and Ellis LM: Regulatory role of c-Met in insulin-like growth factor-I receptor-mediated migration and invasion of human pancreatic carcinoma cells. *Mol Cancer Ther* 5: 1676-1682, 2006.
- Bauer TW, Fan F, Liu W, Johnson M, Parikh NU, Parry GC, Callahan J, Mazar AP, Gallick GE and Ellis LM: Insulinlike growth factor-I-mediated migration and invasion of human colon carcinoma cells requires activation of c-Met and urokinase plasminogen activator receptor. *Ann Surg* 241: 748-758, 2005.
- Cui JJ, Tran-Dubé M, Shen H, Nambu M, Kung PP, Pairish M, Jia L, Meng J, Funk L, Botrous I, *et al*: Structure based drug design of crizotinib (PF-02341066), a potent and selective dual inhibitor of mesenchymal-epithelial transition factor (c-MET) kinase and anaplastic lymphoma kinase (ALK). *J Med Chem* 54: 6342-6363, 2011.
- Iorio F, Knijnenburg TA, Vis DJ, Bignell GR, Menden MP, Schubert M, Aben N, Gonçalves E, Barthorpe S, Lightfoot H, *et al*: A landscape of pharmacogenomic interactions in cancer. *Cell* 166: 740-754, 2016.
- Zhu XD, Zhang JB, Zhuang PY, Zhu HG, Zhang W, Xiong YQ, Wu WZ, Wang L, Tang ZY and Sun HC: High expression of macrophage colony-stimulating factor in peritumoral liver tissue is associated with poor survival after curative resection of hepatocellular carcinoma. *J Clin Oncol* 26: 2707-2716, 2008.
- Lee J, Seo JW, Jun HJ, Ki CS, Park SH, Park YS, Lim HY, Choi MG, Bae JM, Sohn TS, *et al*: Impact of MET amplification on gastric cancer: Possible roles as a novel prognostic marker and a potential therapeutic target. *Oncol Rep* 25: 1517-1524, 2011.
- Ma PC, Tretiakova MS, MacKinnon AC, Ramnath N, Johnson C, Dietrich S, Seiwert T, Christensen JG, Jagadeeswaran R, Krausz T, *et al*: Expression and mutational analysis of MET in human solid cancers. *Genes Chromosomes Cancer* 47: 1025-1037, 2008.
- Zhang H, Pelzer AM, Kiang DT and Yee D: Down-regulation of type I insulin-like growth factor receptor increases sensitivity of breast cancer cells to insulin. *Cancer Res* 67: 391-397, 2007.
- Wolf S, Lorenz J, Mössner J and Wiedmann M: Treatment of biliary tract cancer with NVP-AEW541: Mechanisms of action and resistance. *World J Gastroenterol* 16: 156-166, 2010.
- Kaulfuss S, Burfeind P, Gaedcke J and Scharf JG: Dual silencing of insulin-like growth factor-I receptor and epidermal growth factor receptor in colorectal cancer cells is associated with decreased proliferation and enhanced apoptosis. *Mol Cancer Ther* 8: 821-833, 2009.
- Cunningham MP, Thomas H, Marks C, Green M, Fan Z and Modjtahedi H: Co-targeting the EGFR and IGF-1R with anti-EGFR monoclonal antibody ICR62 and the IGF-1R tyrosine kinase inhibitor NVP-AEW541 in colorectal cancer cells. *Int J Oncol* 33: 1107-1113, 2008.
- Bao XH, Takaoka M, Hao HF, Wang ZG, Fukazawa T, Yamatsuji T, Sakurama K, Sun DS, Nagasaka T, Fujiwara T and Naomoto Y: Esophageal cancer exhibits resistance to a novel IGF-1R inhibitor NVP-AEW541 with maintained RAS-MAPK activity. *Anticancer Res* 32: 2827-2834, 2012.

28. Min Y, Adachi Y, Yamamoto H, Ito H, Itoh F, Lee CT, Nadaf S, Carbone DP and Imai K: Genetic blockade of the insulin-like growth factor-I receptor: A promising strategy for human pancreatic cancer. *Cancer Res* 63: 6432-6441, 2003.
29. Jaquish DV, Yu PT, Shields DJ, French RP, Maruyama KP, Niessen S, Hoover H, A Cheres D, Cravatt B and Lowy AM: IGF1-R signals through the RON receptor to mediate pancreatic cancer cell migration. *Carcinogenesis* 32: 1151-1156, 2011.
30. Prenen H, Tejpar S and Van Cutsem E: New strategies for treatment of KRAS mutant metastatic colorectal cancer. *Clin Cancer Res* 16: 2921-2926, 2010.
31. Bai Y, Kim JY, Watters JM, Fang B, Kinose F, Song L, Koomen JM, Teer JK, Fisher K, Chen YA, *et al*: Adaptive responses to dasatinib-treated lung squamous cell cancer cells harboring DDR2 mutations. *Cancer Res* 74: 7217-7228, 2014.
32. Topcu-Yilmaz P, Kiratli H, Saglam A, Söylemezoglu F and Hascelik G: Correlation of clinicopathological parameters with HGF, c-Met, EGFR, and IGF-1R expression in uveal melanoma. *Melanoma Res* 20: 126-132, 2010.
33. Lennerz JK, Kwak EL, Ackerman A, Michael M, Fox SB, Bergethon K, Lauwers GY, Christensen JG, Wilner KD, Haber DA, *et al*: MET amplification identifies a small and aggressive subgroup of esophagogastric adenocarcinoma with evidence of responsiveness to crizotinib. *J Clin Oncol* 29: 4803-4810, 2011.
34. Jardim DL, de Melo Gagliato D, Falchook GS, Janku F, Zinner R, Wheler JJ, Subbiah V, Piha-Paul SA, Fu S, Murphy MB, *et al*: MET aberrations and c-MET inhibitors in patients with gastric and esophageal cancers in a phase I unit. *Oncotarget* 5: 1837-1845, 2014.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.