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PRECLINICAL STUDIES

Activation of IGF-1R pathway and NPM-ALK G1269A mutation confer resistance to crizotinib treatment in NPM-ALK positive lymphoma

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Summary
ALK-positive anaplastic large cell lymphoma (ALCL) represents a subset of non-Hodgkin's lymphoma that is treated with crizotinib, a dual ALK/MET inhibitor. Despite the remarkable initial response, ALCLs eventually develop resistance to crizotinib. ALK inhibitor resistance in tumors is a complex and heterogeneous process with multiple underlying mechanisms, including ALK gene amplification, ALK kinase domain mutation, and the activation of various bypass signaling pathways. To overcome resistance, multiple promising next-generation ALK kinase inhibitors and rational combinatorial strategies are being developed. To determine how cancers acquire resistance to ALK inhibitors, we established a model of acquired crizotinib resistance by exposing a highly sensitive NPM-ALK-positive ALCL cell line to increasing doses of crizotinib until resistance emerged. We found that the NPM-ALK mutation was selected under intermediate-concentration drug stress in resistant clones, accompanied by activation of the IGF-1R pathway. In the crizotinib-resistant ALCL cell model, the IGF-1R pathway was activated, and combined ALK/IGF-1R inhibition improved therapeutic efficacy. Furthermore, we also detected the NPM-ALK G1269A mutation, which had previously been demonstrated to result in decreased affinity for crizotinib, in the resistant cell model. Although crizotinib was ineffective against cells harboring the NPM-ALK G1269A mutation, five structurally different ALK inhibitors, alectinib, ceritinib, TAE684, ASP3026 and AP26113, maintained activity against the resistant cells. Thus, we have shown that second-generation ALK tyrosine kinase inhibitors or IGF-1R inhibitors are effective in treating crizotinib-resistant tumors.

Keywords ALK · ALCL · Crizotinib · IGF-1R · Drug resistance

Introduction

Anaplastic lymphoma kinase (ALK) was first identified in anaplastic large-cell lymphoma (ALCL) and was one of the few oncogenes activated in both hematopoietic and non-hematopoietic malignancies [1–3]. Approximately 80% patients with ALCL harbor the NPM-ALK fusion, and subsequent studies found various ALK chromosomal translocations in other cancers, such as echinoderm microtubule-associated protein like 4 (EML4)-ALK fusion in non-small cell lung

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cancer (NSCLC) and ran-binding protein 2 (RANBP2)-ALK in inflammatory myofibroblastic tumors [2–6]. In all fusion proteins, the fusion partners act on ALK, resulting in the constitutive activation of ALK kinase, promoting oncogenesis [7–9]. Crizotinib, an ALK tyrosine kinase inhibitor (TKI), has shown remarkable efficacy in ALK+ NSCLC as well as in relapsed ALK+ ALCL patients [10–14]. However, while many ALK+ NSCLC patients initially benefit from crizotinib treatment, drug resistance inevitably arises, leading to disease progression and tumor relapse [15–18].

A variety of mechanisms for the acquired resistance to crizotinib have been described. ALK kinase domain secondary mutations, including L1196 M, G1269A, L1152R, C1156Y, I1171T, F1174 L, G1202R, and S1206Y, have been identified as the key mechanism of resistance to crizotinib [19–24]. The G1269A mutation, in which the glycine at 1269 is substituted with an alanine, causes steric hindrance, resulting in decreased affinity for crizotinib [25, 26]. Additionally, gain in ALK copy number and loss of ALK gene rearrangement have also been implicated in the development of acquired resistance to crizotinib [21–23]. Various second-generation and third-generation ALK inhibitors have been used to overcome resistance to crizotinib in ALK positive ALCLs [27–30]. But the sensitivity of ALK inhibitors differs among different types of NPM-ALK mutations [31–36].

In approximately 40% of patients with crizotinib-resistant tumors, the activation of survival signals through alternative pathways, the so-called “bypass pathway” activation, has been observed [37–41]. Type 1 insulin-like growth factor receptor (IGF-1R), a tetrameric transmembrane receptor tyrosine kinase, binds to its ligand IGF-1, leading to the activation of major signaling pathways including IRS-1/PI3K/AKT, MAPK/ERK, and JAK/STAT [42–44]. Previous studies have reported that IGF-1R and IGF-1 are widely expressed in ALK+ ALCL cell lines and primary tumors [44]. Recently, IGF-1R was shown to be involved in the crizotinib resistance mechanism in ALK+ NSCLC, and the combination of an IGF-1R inhibitor with crizotinib resensitized crizotinib-resistant cells [45–47]. In ALK+ ALCL cells, IGF-1R binds to and activates NPM-ALK, and inhibition of IGF-1R suppresses the growth of ALK+ ALCL cells [44].

Although several mechanisms for crizotinib resistance have been suggested, the specific signaling pathways involved in crizotinib resistance in NPM-ALK+ ALCL have remained unclear. In this study, we explored crizotinib resistance by generating and characterizing a cell line model of acquired crizotinib resistance. In this model, IGF-1R pathway activation and NPM-ALK G1269A mutation conferred resistance to crizotinib. We showed that single-clone cells expressing NPM-ALK with the G1269A mutation were resistant to crizotinib, remained addicted to ALK signaling and were highly sensitive to other structurally distinct ALK tyrosine kinase inhibitors (TKIs). Treatment with an IGF-1R pathway inhibitor also enhances the sensitivity to crizotinib in resistant cells. Based on these results, we propose two therapeutic strategies, IGF-1R pathway inhibitor and second-generation ALK TKIs, for overcoming acquired resistance to crizotinib.

Material and methods

Compounds and cell lines

Crizotinib and picropodophyllotoxin (PPP) were purchased from Bio Vision (Milpitas, CA, USA). Alectinib, ceritinib, TAE684, AP26113 and ASP3026 were purchased from Selleck Chemicals (Houston, TX, USA). Human insulin-like growth factor I was purchased from Cell Signaling Technology (Danvers, MA, USA). The ALK+ ALCL cell line Karpas299 was kindly provided by Dr. Raymond Lai (University of Alberta, Canada) [26, 48]. Karpas299WT, Karpas299CR and Karpas299CR^{G1269A} cells were maintained in RPMI 1640 (Life Technologies, Grand Island, NY, USA) supplemented with 10% FBS and cultured under an atmosphere of 95% O_2 and 5% CO_2 in 98% humidity at 37 °C.

Generation of crizotinib-resistant cell lines

To create crizotinib-resistant lines, crizotinib-sensitive parental Karpas299 cells (Karpas299WT) were cultured in the presence of increasing concentrations of crizotinib starting at 30 nM. Dosage was increased in a stepwise pattern when normal cell proliferation resumed. Fresh drug was added every 48–72 h. Karpass299 crizotinib-resistant (Karpas299CR) cells that grew in 400 nM crizotinib were derived after approximately 4 months of culturing in the continuous presence of drug. DNA identity testing on both the parental and resistant cells confirmed that the cells were derived from the same origin.

Cell viability and drug sensitivity

Cells were seeded at a concentration of 1×10^4 cells/well in round bottom 96-well cell culture plates with complete medium containing various concentrations of inhibitors. MTS (3-(4,5-dimethyl-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2Htetrazolium, inner salt) assay was used to determine the number of viable cells, as an indirect method to assess viability and was performed with the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega), according to the manufacturer’s protocol.

Antibodies and immunoblotting

The following antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA): ALK (catalog number 3333S), phospho-ALK tyrosine 1604 (catalog number

3341 L), IGF-1R (catalog number 3027 L), phospho-IGF-1Receptor β (Tyr1135/1136) (catalog number 3024 L), STAT3 (catalog number 9132 L), phospho-STAT3 (Tyr705) (catalog number 9131 L), ERK (catalog number 4695S), phospho-ERK threonine 202/tyrosine 204 (catalog number 4370 L), AKT (catalog number 2966 L), phospho-AKT serine 473 (catalog number 3787 L), poly(ADP-ribose) polymerase (PARP) (catalog number 9542 L), caspase-3 (catalog number 9662S), caspase-9 (catalog number 9508S), and PUMA (catalog number 12450S). Antibody against actin (catalog number sc-1616) and secondary goat anti-mouse and goat anti-rabbit antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Survivin antibody (catalog number NB100–56167; NOVUS Biologicals, Littleton, CO, USA) was also used.

For immunoblotting, cells were washed twice with phosphate-buffered saline (PBS) and lysed in lysis buffer (1% Triton X-100, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM EDTA, 100 mM NaF, 1 mM Na3VO4, 1 mM PMSF, 2 μg/ml aprotinin). Protein concentrations were quantified using the BCA method (ab102536; Abcam, Cambridge, UK). Protein samples (30 μg total protein/lane) were separated by 8% or 12% sodium dodecyl sulfate poly-acrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Immoblin-P; Millipore, Merck KGaA; Darmstadt, Germany). After blocking the membrane with 5% skim milk in tris-buffered saline Tween-20 (TBST; 10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% Tween 20) at room temperature for 1 h, primary antibodies were added and the membrane was incubated overnight at 4 °C. The following day, membranes were washed three times with TBST and then incubated with secondary goat anti-rabbit or goat anti-mouse antibodies for 30 to 40 min at room temperature, followed by three washes with TBST buffer. The protein bands were detected with enhanced chemiluminescence reagent (SuperSignal™ Western Pico Chemiluminescent Substrate; Pierce, Waltham, MA, USA) and scanned using the Electrophoresis Gel Imaging Analysis System (DNR Bio-Imaging Systems, Neve Yamin, Israel).

Flow cytometry assay

Cells were collected, washed in PBS, and resuspended in 200 μL binding buffer. Next, 5 μL of Annexin V-FITC (BD Biosciences, Franklin Lakes, NJ, USA) was added to 195 μL of cell suspension. After mixing and incubating for 10 min at room temperature, the cells were washed with 200 μL binding buffer and resuspended in 190 μL binding buffer. Then, 10 μL of propidium iodide (20 μg/mL) was added. The samples were then evaluated by flow cytometry (BD Accuri™ C6 Flow cytometer; BD Biosciences) and data were analyzed with WinMDI version 2.9 software (The Scripps Research Institute, La Jolla, CA, USA).

Enzyme-linked immunosorbent assay

Karpas299WT and Karpas299CR cells were seeded in six-well plates and cultured for 24 h. Culture supernatants were collected and the number of cells was counted. IGF-1 levels were analyzed using the Human IGF-1 Quantikine ELISA kit (R&D Systems, DG100) according to the manufacturer's protocol, and data were normalized to total cell counts.

Short interfering RNA and gene transfection

To downregulate the expression of IGF-1R, Karpas299CR cells (5×10^5 cells in 0.5 ml of culture medium) were transfected with 100 pmol of SMARTpool-designed siRNA against IGF-1R. Cells transfected with scrambled siRNA (Dharmacon, Lafayette, CO, USA) were used as negative controls. All siRNA transfection experiments were performed using the BTX ECM 830 square wave electroporator (BTX, Holliston, MA, USA) at 225 V (8.0 ms pulse length, 3 pulses, 1 s between pulses).

Statistical analysis

Data were analyzed using SPSS version 21.0 software (IBM, Armonk, NY, USA). Data were expressed as the mean ± standard deviation. Differences of the results between the two groups were determined by Student's t tests. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Generation and characterization of crizotinib-resistant cells

The ALCL cell line Karpas299 expresses NPM-ALK and is highly sensitive to crizotinib treatment. To explore the mechanisms of crizotinib resistance, we generated a crizotinib-resistant Karpas299 cell line by culturing the cells in the presence of increasing concentrations of crizotinib for 4 months. We maintained the crizotinib-resistant Karpas299 (Karpas299CR) cells in 400 nM of crizotinib. MTS assays confirmed that Karpas299CR cells were resistant to crizotinib, whereas the parental Karpas299WT cells were not (Fig. 1a).

Activation of the IGF-1R pathway and G1269A mutation coexist in crizotinib-resistant cells

Previous studies have revealed that the mechanisms of resistance to crizotinib include secondary mutations within the

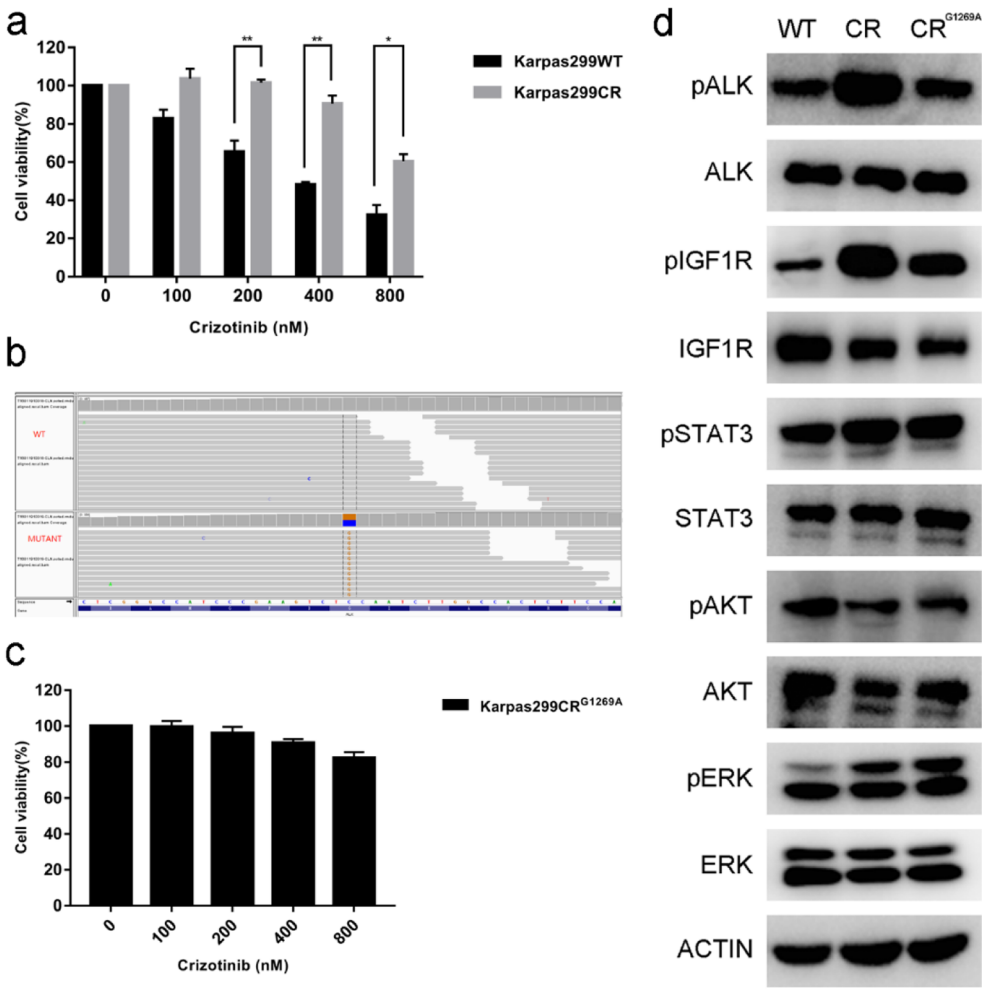

Fig. 1 Karpas299CR and Karpas299CR^{G1269A} cells are resistant to crizotinib. **a**, Cell lines were seeded in 96-well plates and treated with various concentrations of crizotinib for 48 h. Cell viability was analyzed using the MTS assay. Data are presented as the mean ± standard deviation of three independent experiments. **b**, Karpas299CR and Karpas299CR^{G1269A} cells were analyzed using ultra-deep next generation sequencing to detect the abundance of ALK mutations. **c**, Karpas299CR^{G1269A} cells were treated with various crizotinib concentrations for 48 h. Cell viability was analyzed using the MTS assay. **d**, Immunoblotting of the indicated proteins in the parental and in two crizotinib-resistant cell lines. Equal volumes of DMSO were added as the negative control. *$P < 0.05$, **$P < 0.01$

ALK kinase domain, ALK copy number amplification, the loss of ALK gene rearrangement and the activation of alternative signaling. We first examined whether resistant lines might harbor an activating mutation within the ALK kinase domain by analyzing the mutation status of ALK in parental and resistant cells by ultra-deep next generation sequencing. In Karpas299CR cells, we observed a C → G substitution resulting in the change from glycine to alanine at position 1269 within a mutation rate of 49.79% (Fig. 1b). Previous studies reported that this 1269 C → G substitution results in decreased affinity of ALK for crizotinib. We next generated Karpas299CR single-cell subclones by limiting dilution method and selected a G1269A-mutated subline for further study, which we named Karpas299CR^{G1269A}.

To examine the resistance of Karpas299CR^{G1269A} to crizotinib, we tested the effect of crizotinib on the cell growth rate and phosphorylation status of ALK and its downstream effectors. Cell viability assays confirmed that the Karpas299CR^{G1269A} cell line was resistant to crizotinib (Fig. 1c). Consistent with the cell viability results, crizotinib treatment had limited impact on the proliferation of resistant cell lines. Crizotinib treatment of Karpas299CR^{G1269A} cells up to 800 nM did not cause any changes in cell proliferation.

To determine whether the acquired resistance of Karpas299CR cells is due to the induction of alternative signaling pathways, we analyzed several pathways by western blot. Both resistant cell lines showed a significant increase in ALK phosphorylation status compared to parental cells, and a corresponding increase in the phosphorylation status of IGF-1R and its downstream proteins such as STAT3 or ERK, was also observed in resistant cell lines. However, the phosphorylation of ALK and IGF-1R in Karpas299CR^{G1269A} cells was not as high as that of Karpas299CR cells. The same trend was observed in the phosphorylation of ERK and STAT3 (Fig. 1d).

Crizotinib-resistant cells maintain survival and apoptosis resistance compared to parental cells

Previous studies have shown that the deregulated kinase activity of ALK leads to the activation of several downstream pathways such as MEK/ERK, STAT and PI3K/AKT pathways, which result in abnormal proliferation and suppression

of apoptosis. We performed western blot analysis on lysates from parental and crizotinib-resistant cells treated with either DMSO or increasing concentrations of crizotinib. In Karpas299WT cells, crizotinib reduced IGF-1R, STAT3, AKT and ERK phosphorylation in a dose-dependent manner, but these effects were not observed in Karpas299CR and Karpas299CR^{G1269A} cells (Fig. 2a). The phosphorylation of ALK and its crucial downstream signaling intermediates ERK and STAT3 were maintained in the resistant cell lines even at substantially high doses of crizotinib compared to parental cells.

Mitochondrial BCL-2 family proteins have also been implicated in the regulation of ALCL cell survival. Therefore, we also examined changes in the DNA damage-associated protein PARP, pro-apoptotic protein PUMA, and anti-apoptotic protein survivin as well as caspase-3 and caspase-9 cleavage in response to crizotinib treatment. In Karpas299WT cells, survivin was robustly downregulated after 24 h of treatment with crizotinib, while significant increases in PARP, PUMA, caspase-3 and caspase-9 cleavage in response to crizotinib were observed (Fig. 2b). These changes were not seen in Karpas299CR and Karpas299CR^{G1269A} cells.

To further study the biological effects of the inhibition of NPM-ALK on the growth and survival of ALCL cells, we examined the level of apoptosis in cells treated with either crizotinib or DMSO by flow cytometry. In Karpas299WT cells treated with crizotinib for 24 h, approximately 15–30% of the cells were Annexin V-positive (Fig. 2c). In contrast,

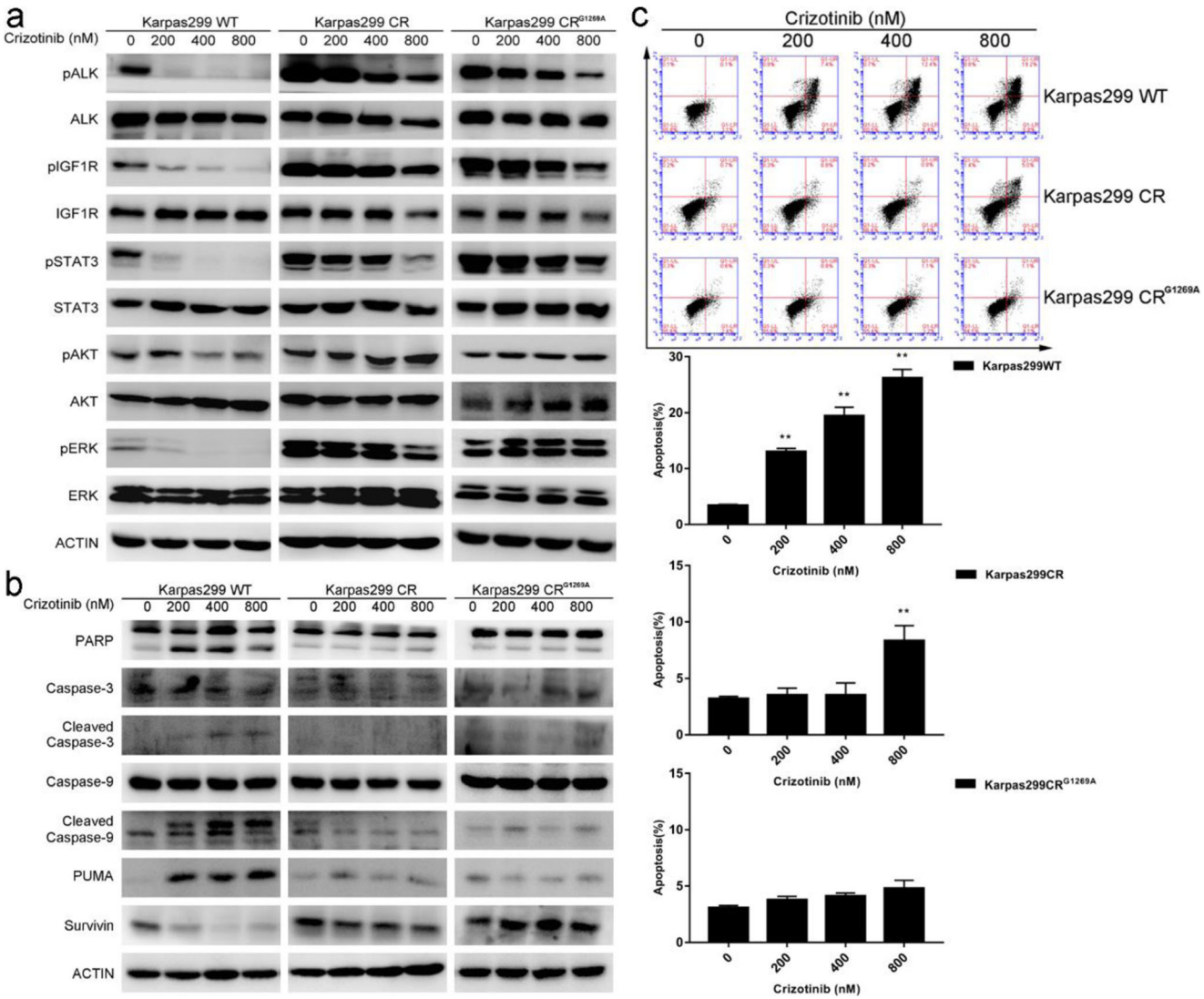

Fig. 2 Sensitivity of Karpas299WT and Karpas299 crizotinib-resistant cells to crizotinib. **a**, Cells were treated with crizotinib at the indicated concentrations for 24 h. Immunoblotting was performed for the indicated proteins. **b**, Cells were treated with crizotinib at the indicated concentrations for 24 h and levels of apoptosis-related proteins were assayed by western blot analysis. **c**, Cells were treated with various concentrations of crizotinib for 24 h and then subjected to apoptosis analysis by flow cytometry. Equal volumes of DMSO were added as the negative control. $^{*}P < 0.05$, $^{**}P < 0.01$

no significant increase in the number of Annexin V-positive cells was seen for Karpas299CR^{G1269A} cells treated with crizotinib compared to the controls. In Karpas299CR cells, only 8% of the cells were Annexin V-positive when treated with 800 nM crizotinib.

Additive effects of crizotinib and IGF-1R inhibitor in Karpas299CR cells but not Karpas299^{G1269A} cells

To assess if IGF-1R was activated by IGF-1 in the Karpas299 cell line, we examined the secretion of IGF-1 in Karpas299 parental and crizotinib-resistant cells. The secretion level of IGF-1 in Karpas299CR cells was increased by more than three-fold (Fig. 3a). IGF-1 cotreatment resulted in protection against the growth inhibitory effects of crizotinib in Karpas299WT cells (Fig. 3b). Furthermore, stimulation of crizotinib-treated Karpas299WT cells with IGF-1 resulted in sustained downstream signaling actication of IGF-1R, as evidenced by the phosphorylation of STAT3, AKT and ERK (Fig. 3c). To assess if the ligand induced activation of IGF-1R could influence the anti-proliferative effects of ALK blockade, IGF-1R knock-down sensitized Karpas299CR cells to the effects of crizotinib (Fig. 3d). Taken together, these data suggest that IGF-1 activated IGF-1R resulting in resistance to crizotinib in Karpas299CR cells.

Next, we tested the ability of an IGF-1R inhibitor, alone or combined with crizotinib, to impede the growth of crizotinib-resistant cells. The IGF-1R inhibitor, picropodophyllotoxin, shows moderate single agent activity in ALCL cells. The combination of crizotinib and picropodophyllotoxin partially restored crizotinib sensitivity in Karpas299CR cells (Fig. 4a). Furthermore, crizotinib and picropodophyllotoxin combination treatment inhibited the phosphorylation of ALK, IGF-1R, STAT3, AKT, and ERK to a greater extent than either inhibitor alone (Fig. 4b). Together these results indicate that the addition of picropodophyllotoxin partially restored the sensitivity of Karpas299CR cells to the growth inhibitory effects of crizotinib.

In addition, we examined whether the NPM-ALK G1269A mutation or IGF-1R pathway activation was the main cause of crizotinib resistance in Karpas299CR^{G1269A} cell lines. We found no significant difference between the proliferation of Karpas299CR^{G1269A} cells treated with crizotinib alone compared to the combination of crizotinib with picropodophyllotoxin (Fig. 4c). Western blot analysis showed that the combination treatment with crizotinib and picropodophyllotoxin did not have

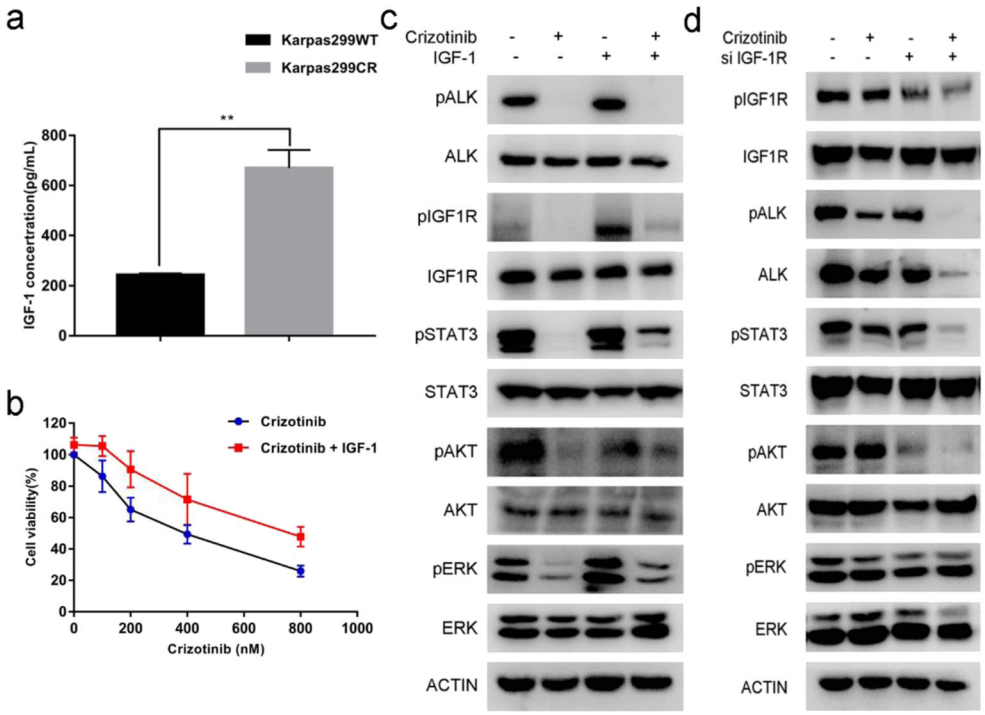

Fig. 3 IGF-1 impairs downstream signaling and promotes proliferation of ALK+ ALCL cells. **a**, IGF-1 concentrations in the supernatants of cultured Karpas299WT and Karpas299CR cells were assessed after 24 h by ELISA. **b**, Karpas299WT cells were treated with the indicated concentrations of crizotinib or crizotinib +100 ng/mL IGF-1 for 72 h. MTS assays were performed to assess growth inhibition. Data are presented as the percentage of viable cells compared to the control. **c**, Karpas299WT cells were serum starved overnight and then treated with crizotinib for 24 h. Cells were then stimulated with IGF-1 for 10 min as indicated. Lysates were subjected to immunoblotting with antibodies for the indicated proteins. **d**, Karpas299CR cells were transfected with the non-targeting siRNA ("NC") or IGF-1R siRNA and combined with crizotinib for 36 h. Lysates were subjected to immunoblotting with antibodies specific for the indicated proteins. Equal volumes of DMSO were added as the negative control. $^{*}P < 0.05$, $^{**}P < 0.01$

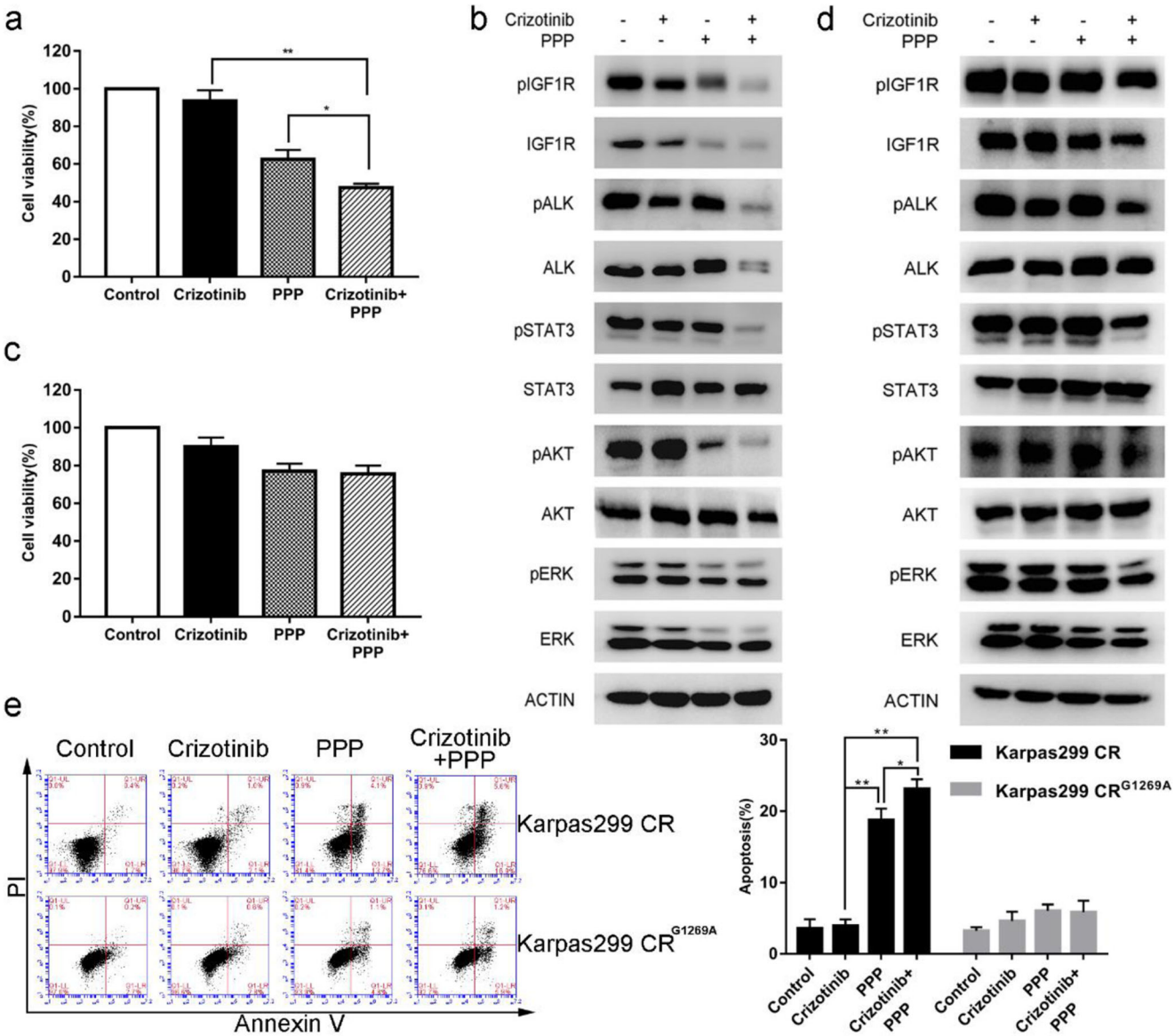

Fig. 4 Combination treatment with the IGF-1R inhibitor PPP plus crizotinib promotes cooperative inhibition of cell growth in Karpas299CR cells. **a**, Karpas299CR cells were treated with 400 nM crizotinib or 400 nM crizotinib +1.0 μM PPP for 24 h, and cell viability was analyzed by MTS assays. **b**, Karpas299CR cells were treated with 400 nM crizotinib, 1.0 μM PPP, or the combination and then examined by immunoblotting for the indicated proteins. **c**, Karpas299CR^{G1269A} cells were treated with 400 nM crizotinib, 1.0 μM PPP, or the combination for 24 h and cell viability was analyzed by MTS assays. **d**, Karpas299CR^{G1269A} cells were treated with 400 nM crizotinib, 1.0 μM PPP, or the combination, and lysates were subjected to immunoblotting with antibodies specific for the indicated proteins. e, Karpas299CR and Karpas299CR^{G1269A} cells were treated with 400 nM crizotinib, 1.0 μM PPP, or the combination for 24 h, and apoptosis rates were detected by flow cytometry. Equal volumes of DMSO were added to the control group as the negative control. PPP, picropodophyllotoxin. $*P < 0.05$, $**P < 0.01$

the same impact as observed in Karpas299CR cells (Fig. 4d). Furthermore, the combination of IGF-1R inhibitor plus crizotinib resulted in more significant inhibition of growth and apoptosis in Karpas299CR cells (Fig. 4e). These results demonstrated that crizotinib-resistant Karpas299CR^{G1269A} cells remain addicted to ALK signaling and picropodophyllotoxin could not restore crizotinib sensitivity in these cells. This observation suggests that IGF-1R signaling operates as a resistance mechanism in Karpas299CR but not Karpas299CR^{G1269A} cells.

Second-generation ALK inhibitors overcome crizotinib resistance

Although both Karpas299CR and Karpas299CR^{G1269A} cells showed resistance to crizotinib, the two cell lines may be sensitive to structurally distinct ALK kinase inhibitors. Thus, we examined the effects of 5 second-generation ALK inhibitors, alectinib, ceritinib, TAE684, ASP3026 and AP26113, on cell proliferation. As expected,

Table 1 IC_{50}values of ALK inhibitors in Karpas299WT, Karpas299CR and Karpas299CR^{G1269A} cell lines

	IC_{50} (μmol/L)					
Cell lines	Crizotinib	Alectinib	Ceritinib	TAE684	ASP3026	AP26113
Karpas299 WT	0.290	0.147	0.019	0.010	0.136	0.009
Karpas299 CR	0.724	0.258	0.022	0.006	0.163	0.015
Karpas299 CR^{G1269A}	2.945	0.669	0.017	0.012	0.316	0.012

all 5 second-generation ALK inhibitors were effective in inhibiting crizotinib-resistant cell proliferation and with lower IC_{50} values compared to crizotinib (Tables 1 and 2). However, alectinib and ASP3026 had a weaker inhibition on resistant cells compared to the other three inhibitors, especially in Karpas299CR^{G1269A} cells. To confirm these data, we investigated the ALK, STAT3, AKT and ERK phosphorylation status in crizotinib-resistant cell lines treated with each of the five inhibitors. ALK phosphorylation and downstream signaling were completely abrogated or strongly inhibited in Karpas299CR cells treated with each of the five inhibitors (Fig. 5a–e). However, alectinib and ASP3026 did not display the same degree of activity in Karpas299CR and Karpas299CR^{G1269A} cells. We suspect that this phenomenon is caused by the presence of ALK G1269A mutation.

Dual ALK/IGF-1R inhibition represses cell proliferation and cell signaling pathways in Karpas299CR cells but not Karpas299^{G1269A} cells

AP26113 and certinib are second-generation TKIs against ALK and show selectivity against IGF-1R. Since Karpas299CR^{G1269A} cells were more susceptible to the ALK G1269A mutation than IGF-1R signaling, AP26113 showed more significant inhibition of viability in Karpas299CR cells than in Karpas299CR^{G1269A} cells when combined with the IGF-1R inhibitor picropodophyllotoxin (Fig. 6a, b). The same phenomenon was observed when combining ceritinib with picropodophyllotoxin (Fig. 6c, d). Western blot analysis in Karpas299CR cells showed that combination treatment with the ALK inhibitors and picropodophyllotoxin inhibited IGF-1R, AKT, and ERK phosphorylation to a greater extent than either inhibitor alone (Fig. 6e, g). However, in Karpas299CR^{G1269A} cells, the combination treatment did not have the same effect as in Karpas299CR cells (Fig. 6f, h).

Discussion

ALK+ ALCL therapy has benefited from the development of crizotinib, the first ALK inhibitor to be successfully used in patients. The development of second-generation ALK inhibitors such as alectinib and ceritinib was aimed to improve treatment efficacy and overcome crizotinib resistance in patients. Combination strategies using two drugs have been widely explored in recent years as an effective approach to overcome resistance to a targeting therapeutic agent.

IGF-1R is widely expressed in ALK+ ALCL cell lines and primary tumors and plays important roles in cell proliferation, apoptosis, cell cycle and migration [44]. In the present work, we generated a human ALK+ ALCL cell line resistant to crizotinib. We found that IGF-1 secretion levels were increased more than three-fold in Karpas299CR cells compared to parental Karpas299WT cells. IGF-1 induced the activation of IGF-1R, which, in turn, led to crizotinib resistance in the cell lines. The ALK inhibitor crizotinib combined with the IGF-1R inhibitor picropodophyllotoxin cooperatively decreased the proliferation and promoted the apoptosis of Karpas299CR cells. To evaluate the effect of the combined treatment on the ALK signaling cascade, we analyzed the phosphorylation status of some known targets of the IGF-1R pathway. The combination treatment of Karpas299CR cells resulted in greater reduction of p-

Table 2 IC_{50} values in Karpas299CR and Karpas299CR^{G1269A} cell lines compared to the parental line calculated from data in Table 1

	IC_{50} Fold Change					
Cell lines	Crizotinib	Alectinib	Ceritinib	TAE684	ASP3026	AP26113
Karpas299 WT	1.0	1.0	1.0	1.0	1.0	1.0
Karpas299 CR	2.5	1.8	1.2	0.6	1.2	1.7
Karpas299 CR^{G1269A}	10.2	4.6	0.9	1.2	2.3	1.3

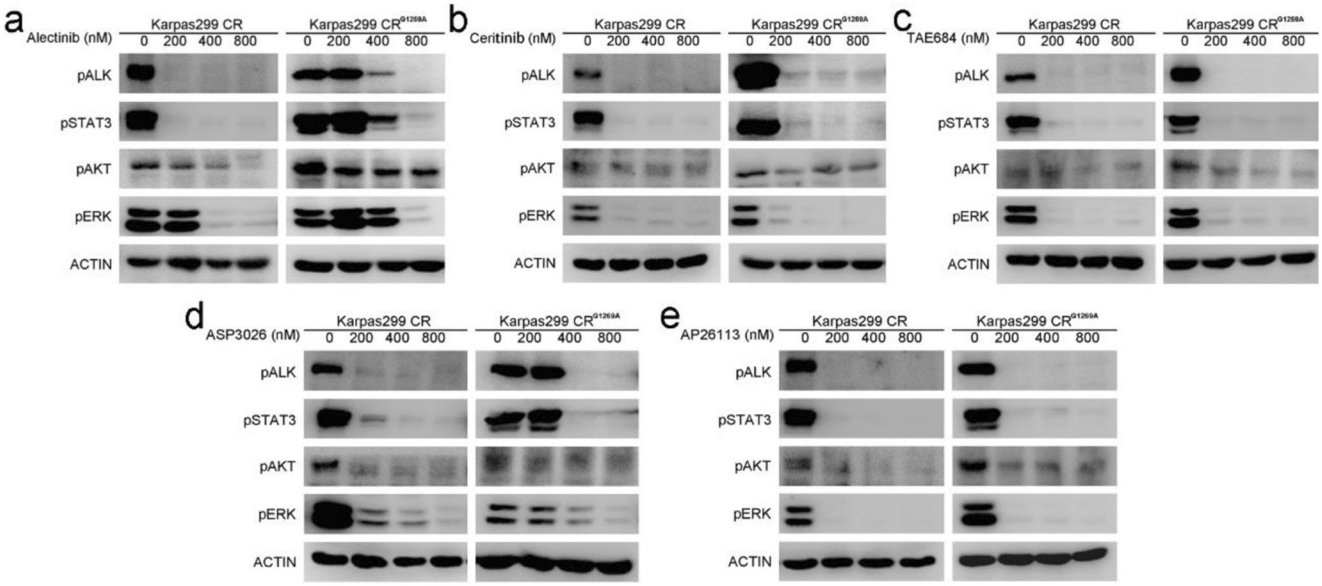

Fig. 5 Second-generation ALK inhibitors inhibit downstream signaling of ALK in Karpas299CR and Karpas299CR^{G1269A} cells. **a** – **e** Karpas299CR (left panels) and Karpas299CR^{G1269A} cells (right panels) were treated with Alectinib (**a**), Ceritinib (**b**), TAE684 (**c**), ASP3026 (**d**) or AP26113 (**e**) at the indicated concentrations for 24 h and examined by immunoblotting.

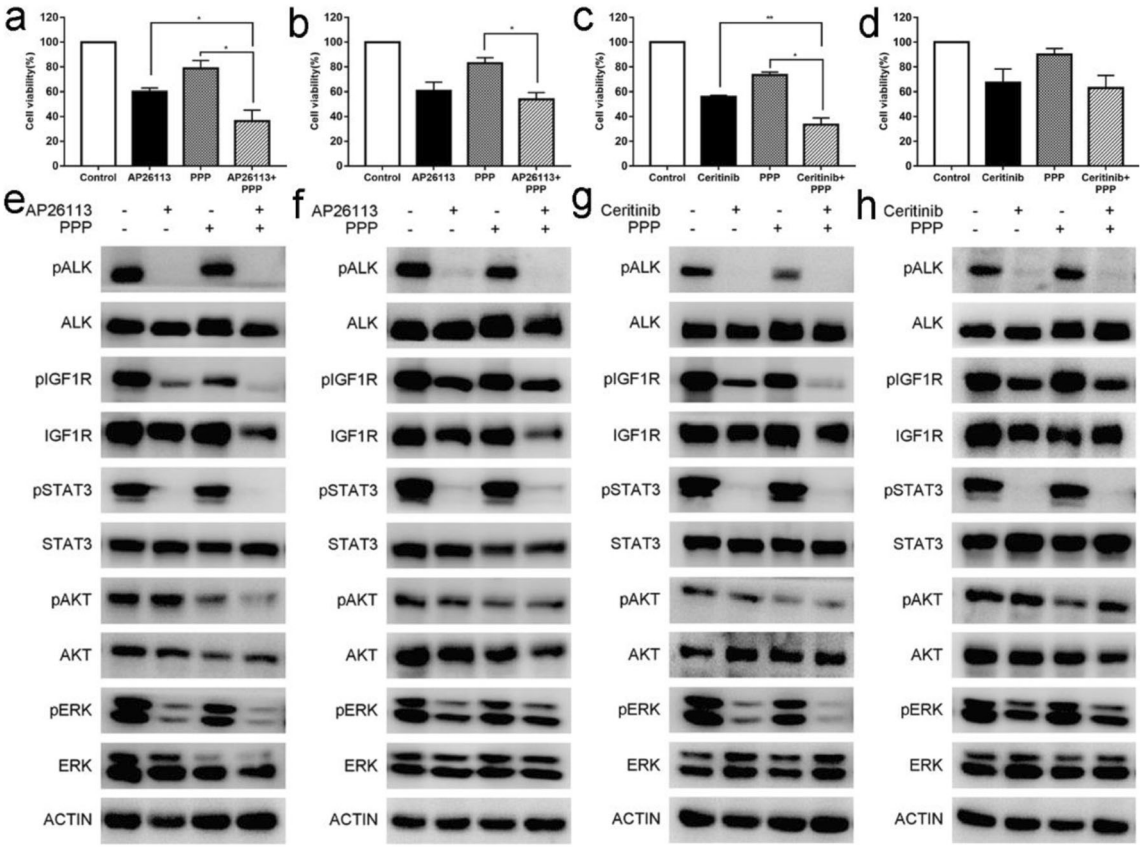

Fig. 6 Combination therapy of the IGF-1R inhibitor PPP with ALK inhibitor promotes cooperative inhibition of cell growth in Karpas299CR cells. Karpas299CR cells were treated with AP26113, PPP or the combination for 24 h and then analyzed by MTS assays (**a**) and immunoblotting (**e**). Karpas299CR^{G1269A} cells were treated with AP26113, PPP or the combination for 24 h and then analyzed by MTS assays (**b**) and immunoblotting (**f**). Karpas299CR cells were treated with Ceritinib, PPP or the combination for 24 h and then analyzed by MTS assays (**c**) and immunoblotting (**g**) Karpas299CR^{G1269A} cells were treated with Ceritinib, PPP or the combination for 24 h and then analyzed by MTS assays (**d**) and immunoblotting (**h**). In all experiments, equal volumes of DMSO were added to the control group as the negative control. PPP, picropodophyllotoxin. $^{*}P < 0.05$, $^{**}P < 0.01$

AKT, p-ERK and p-STAT3 levels compared to single treatments. Importantly, the addition of an IGF-1R inhibitor sensitized the resistant cells to the effects of the ALK blockade. Based on these results, we propose the combination of agents targeting ALK and IGF-1R as a novel therapeutic approach in patients with ALK+ ALCL.

In our study, we found that crizotinib resistance was also mediated by the ALK G1269A mutation in the subclone Karpas299CR^{G1269A} cells. Crizotinib combined with picropodophyllotoxin had no significant impact on proliferation compared to the effect of crizotinib alone.

Resistance to crizotinib in both Karpas299CR and Karpas299CR^{G1269A} cells could be effectively overcome by second-generation ALK inhibitors. The second-generation ALK inhibitors alectinib, ceritinib, TAE684, ASP3026 and AP26113, inhibited the proliferation of crizotinib-resistant cells. Furthermore, second-generation ALK inhibitors AP26113 and ceritinib combined with the IGF-1R inhibitor cooperatively decreased the proliferation and promoted the apoptosis of Karpas299CR cells. The same effect was not observed in Karpas299CR^{G1269A} cells.

Consistent with clinical cases and drug doses, Karpas299CR cells represent the majority of patients during drug treatment. For these patients, the combination of ALK inhibitors and IGF-1R inhibitor may be the best choice to overcome resistance. However, for patients with G1269A-mutated tumors, second-generation TKIs alone should be the most appropriate treatment strategy compared to the combination with an IGF-1R inhibitor.

We describe here for the first time the in vitro efficacy of combined ALK/IGF-1R inhibition for the treatment of ALK+ ALCL. These findings provide a first indication that the combination of low-dose ALK and IGF-1R inhibitors may be beneficial for the treatment of this disease. Moreover, for mutated resistant cells under drug pressure, second-generation TKIs alone may have a larger impact on overcoming resistance than a combination of IGF-1R inhibitors.

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Compliance with ethical standards

Conflict of interests The authors have declared that no competing interest exists.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent For this type of study, formal consent is not required.

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