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Original Article

The effects of the combination of imatinib and crizotinib on small cell lung cancer cells expressing c-Met and c-Kit

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Abstract: Objective: To investigate the effects of the combination of the drug imatinib (inhibiting msenchymal-epithelial transition factor (c-Met) and the drug crizotinib targeting c-kitproto-oncogeneprotein (c-Kit)) on small cell lung cancer (SCLC). Methods: Those effects were investigated with cell counting kit-8 (CCK-8), quantitative PCR (qPCR), western blot (WB), and immunohistochemistry assays. Results: Among the five kinds of SCLC cell lines (H446, H1048, H209, H526, and H889), H526 proliferated fastest and H209 and H889 were slowest. Among the five kinds of SCLC cell lines, the H526 cells had the most cellular abundance for both c-Met and c-Kit. The combination of imatinib and crizotinib was found to have more significant inhibitory effects on the proliferation of H526 cells than imatinib alone or crizotinib alone. In addition, the combination had a more significant ability to decrease the volume of tumors induced by the H526 cells than did the H446 cells, whose cellular abundance of c-Met and c-Kit was lower than the H526 cells. In summary, the combination of imatinib and crizotinib demonstrated a stronger inhibitory effect than imatinib alone or crizotinib alone on the proliferation of SCLC cells, and the scales of the inhibitory effects were associated with the cellular levels of c-Met and c-Kit. Conclusions: This study provides data about a potentially new treatment strategy targeting c-Kit and c-Met for SCLC.

Keywords: Small cell lung cancer (SCLC), c-Met, c-Kit, imatinib, crizotinib

Introduction

SCLC, accounting for 20~25% of all lung cancer cases, is highly aggressive and mainly occurs in smokers [1]. Although SCLC responds well to radiation therapy and chemotherapy, only about 20% of the patients with limited-stages respond well to treatments, and less than 5% of patients with extensive-stages are still alive 5 years after their diagnoses [2-4]. So, the current treatments for SCLC are unsatisfactory, and new and effective treatments are needed.

c-Met, which is also known as MET or hepatocyte growth factor receptor (HGFR), is encoded by the c-Met proto-oncogene [5]. c-Met belongs to the receptor tyrosine kinase (RTK) family, and its only known ligand is the hepatocyte growth factor (HGF). Via the HGF/c-Met signaling pathway, c-Met can induce several biological reactions that give rise to invasive growth [6]. Aberrantly active c-Met is associated with tumor occurrence, growth and transformation [7]. Many studies have shown that c-Met is expressed in numbers of solid tumors, such as hepatocelluar carcinoma and breast carcinoma [8, 9]. Overexpression of c-Met occurs in about 40% of lung cancers [10]. Moreover, it has been reported that the overexpression of c-Met and the activaton of mutations of c-Met may contribute to the invasive growth of non-small cell lung cancer (NSCLC) [11]. c-Met mutations in the DNA sequence encoding novel juxtamembrane domain may regulate the cytoskeletal function of SCLC [12]. Moreover, the HGF/c-Met function in SCLC probably provides a possible therapeutic target [13].

The c-Kit receptor (CD117) is a transmembrane protein with tyrosine kinase activity that also belongs to the RTK family. Generally, c-Kit combined with its ligand stem cell factor (SCF) can activate several downstream signal transduction pathways regulating a variety of cell func-

tions, such as cell proliferation, differentiation and apoptosis and so on [14]. Many studies have demonstrated that the abnormal expression of c-Kit is an important factor in the progression of diseases. Deficiency in function of c-Kit can trigger autosomal mottle disease [15]. Overexpression of c-Kit can be detectable in gastrointestinal tumors (GIST), mast cell tumor and T-cell lymphomas [16, 17]. Furthermore, the high positive rate of c-Kit in GIST made c-Kit become a key indicator for GIST diagnosis [18]. Li *et al*. (2007) demonstrated that 69.2% of SCLC organizations express the c-Kit receptor, where exon 9 and exon 11 mutations are detectable [19].

The discovery of the tyrosine kinase inhibitor is a great breakthrough for tumor therapy. It has been proved that imatinib is effective in treating a number of c-Kit-related diseases [20, 21]. Additionally, crizotinib (PF-02341066), which targets ALK, c-Met, and RON, has been applied to the treatment of NSCLC caused by ALK gene rearrangement [22].

Consequently, we hypothesized that a combined therapy targeting c-Met and c-Kit could have a great potential in treating tumors with a high abundance of c-Met and c-Kit, and this study aims to confirm the above hypothesis.

Materials and methods

Reagents

Anti-c-Met antibody, anti-c-Kit antibody, anti-p-c-Met antibody, anti-p-c-Kit antibody and HRP-conjugated anti-mouse antibody were obtained from Abcam (Cambridge, U.K). Mouse monoclonal antibody against GAPDH was obtained from R&D Systems (Minneapolis, USA).

Cell culturing

Five SCLC cell lines (H446, H1048, H209, H526, and H889) were obtained from the American Type Culture Collection (ATCC, Manassas, USA). All the cell lines were cultured in RPMI-1640 (Invitrogen, Massachusetts, USA) supplemented with 10% (v/v) fetal bovine serum (Invitrogen, Massachusetts, USA) and 1% (v/v) penicillin/streptomycin (Invitrogen, Massachusetts, USA), at 37°C and 5% CO_2 in a carbon dioxide incubator.

Cell proliferation assay

The effects of imatinib (Gleevec®, Pharmaceuticals, Novartis, Basel, Switzerland) and crizotinib (Xalkoril®, Pfizer, New York, USA) on the cell proliferation of SCLC were measured using a CCK-8 assay. 10^4 cells (100 ul) were inoculated into each cell well on the 96-well plate and treated with imatinib alone (47.3 μM), crizotinib alone (16.7 μM), and their combination. Cell proliferation was measured at 24 h after the treatments: 10 ul of the solution containing WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) was added into the cell well according to the manufacturer's instructions, and the optical absorbance was measured at 450 nm after 2 h. All the experiments were performed in triplicate.

RNA extraction and qPCR assay

Total RNA was extracted from the cells with RNAiso plus reagent (TAKARA, Dalian, China) following the manufacturer's instructions. The concentration of RNA was determined by a spectrophotometer. First-strand cDNA was synthesized using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Manheim, Germany). The amplification conditions were as follows: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 58°C for 10 s, and 72°C for 10 s. The amplification was conducted using a 7500 Fast Real-time quantitative PCR System (Applied Biosystems, Invitrogen, Massachusetts, USA). The primer sequences for qPCR of c-Met were 5'-CAGGCAGTGCAGCATGTAGT-3' and 5'-GATGATTCCCTCGGTCAGAA-3'; the sequences for c-Kit were 5'-CTTGCATCCAACTCCAGGAT-3' and 5'-GCAGAATTGGAGAAGCCTTG-3'; the sequences for the internal reference GAPDH were 5'-AATCCCATCACCATCTTCC-3' and 5'-CATCACGCCACAGTTTCC-3'. The mRNA expressions were determined using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

The cells were lysed with a lysis buffer and the lysate was centrifuged under 10000 g at 4°C for 30 min after incubation on ice for 50 min. Then the total protein concentration was measured with a BCA kit and the lysate with an equal total protein amount was loaded and electrophoretically separated on 12% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then the separated proteins were transferred to polyvinylidene fluoride (PVDF) transfer membranes (Amersham HybondTM-P, Massachusetts, USA). After being blocked in 5% (w/v) skim milk, the mem-

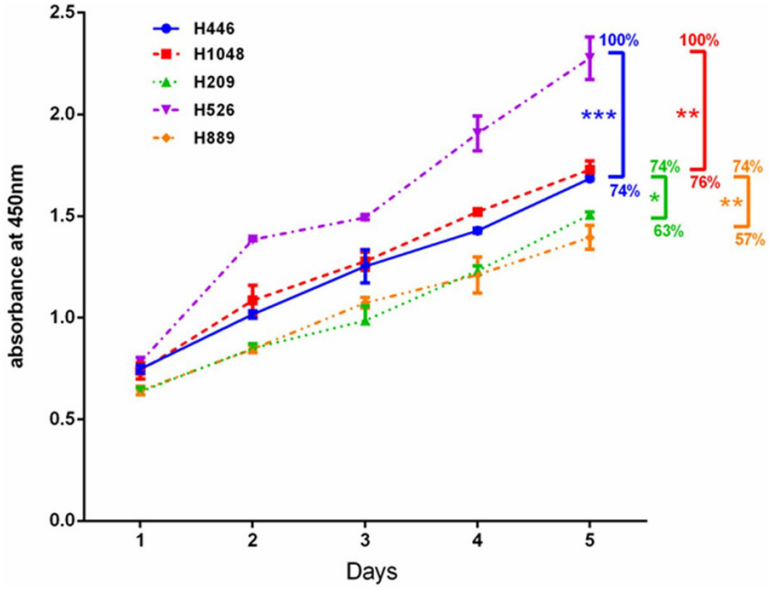

Figure 1. The cell proliferation curves of different SCLC cell lines. The cell proliferation rates of different SCLC cell lines (H446, H1048, H209, H526, and H889) were measured with a CCK-8 assay. After adding WST-8, the cell viability in each well was determined by measuring the optical absorbance at 450 nm. Each data point represents three independent experiments and the data are presented as the means ± SD. The Data were then compared using the LSD method (Least significant method) in a two-way ANOVA analysis. *P < 0.05, **P < 0.01, ***P < 0.001.

branes were incubated with the anti-c-Met antibody (1:1000), the anti-c-Kit antibody (1:1000), or the anti-GAPDH antibody (1:2000), and then incubated with the second antibody. Finally, the protein amounts were measured by chemiluminescence (PerkinElmer Life and Analytical Sciences, Massachusetts, USA).

Tumor xenograft studies

The SCLC cells were amplified, harvested, and resuspended in serum-free media, and 5×10^6 cells were injected subcutaneously under the back skin of 6-week-old female BALB/c nude mice. When the mean tumor volume was approximately 100 mm^3, the mice were treated with imatinib alone (400 mg/kg), crizotinib alone (100 mg/kg), or their combination (200 mg/kg imatinib and 50 mg/kg crizotinib) formulated in saline by intraperitoneal injection. Tumor volume and body weight were measured twice every week. The tumor volume was estimated using the formula: volume = $l \times w^2 \times 0.536$ (w are perpendicular measured diameters) [23]. All the treatments on the animals were in accordance with ethical standards.

Immunohistochemistry

Mice injected with H526 cells were sacrificed at the 17th day after the treatment, the tumor tissues were separated and sliced, and the sections were treated with sodium citrate buffer and incubated with a primary antibody at 4°C overnight. Next, the sections were treated with goat anti-rabbit IgG with horseradish peroxidase (HRP), stained with hematoxylin and then observed under a microscope. Multiple randomly selected microscopic visual fields were used for counting. The relative protein levels of p-c-Kit and p-c-Met were quantified using IPP6.0 software according to the photographs from the immunohistochemistry.

Statistical analysis

The data are presented as the means ± standard deviation (SD) and represent three independent experiments and were analyzed by the LSD method (Least significant method) in One-way or Two-way ANOVA assays. The transcriptional and protein expression levels represented the relative quantification using GAPDH as a reference and were presented as the mean ± SD. Significance was defined as $P < 0.05$.

Results

The correlation of cell proliferation and the expression of c-Met and c-Kit in SCLC cell lines

c-Met and c-Kit play important roles in cancer cells and probably contribute to cell proliferation. To investigate whether a correlation exists between cell proliferation and the expression of c-Kit and c-Met, five SCLC cell lines (H446, H1048, H209, H526, and H889) were studied in this research, where cell proliferation was measured with a CCK-8 assay and the transcriptional and protein expression levels of c-Met and c-Kit were determined by qPCR and WB assays. Data showed that cell proliferation was different in these five SCLC cell lines, and

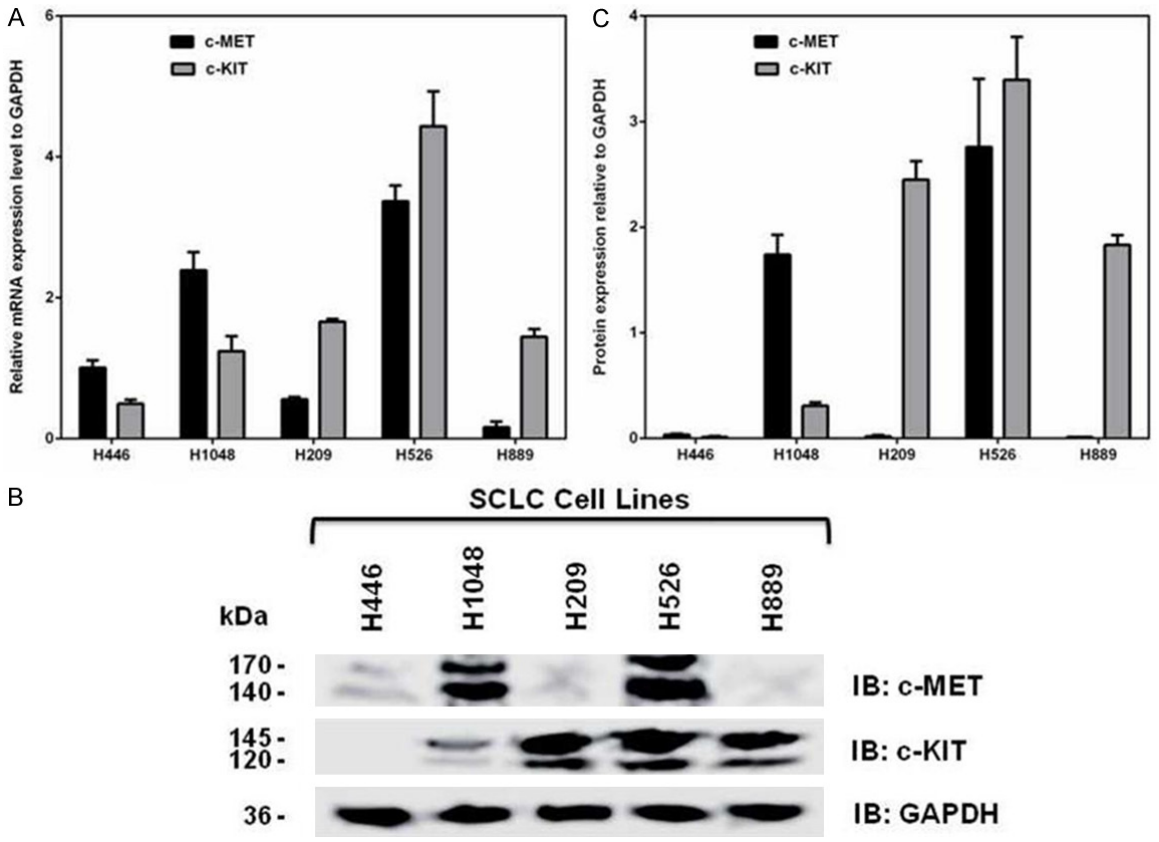

Figure 2. The expression of c-Kit and c-Met in SCLC cell lines. A. The mRNA expressions of c-Met and c-Kit in five SCLC cell lines (H446, H1048, H209, H526, and H889) measured by qPCR. B. The protein expression of c-Met and c-Kit in five SCLC cell lines (H446, H1048, H209, H526 and H889) measured by WB assay. C. The quantification of the protein expression of c-Met and c-Kit in five SCLC cell lines. The grayscale of the protein bands of c-Met and c-Kit was measured by Quantity One based on densitometry, and the data are presented as the means ± SD. GAPDH was used as the reference for the above mRNA and protein measurement. Three independent experiments were conducted, and one representative experiment is presented here.

the proliferation rate of H526 was the highest, and that of H446 was significantly lower (**Figure 1**). Moreover, it was found that the mRNA and protein expression levels of c-Met and c-Kit were highest in H526 and lowest in H446 (**Figure 2**). Data indicated that a positive correlation probably existed between cell proliferation and the expression of c-Kit and c-Met.

Effects of the combinative treatment of imatinib and crizotinib on the proliferation of H526 cells

To investigate whether the combination of imatinib and crizotinib has a stronger inhibition than imatinib alone or crizotinib alone, the proliferation of H526 cells was measured under the treatment of imatinib alone, crizotinib alone, and their combination. Data demonstrated that imatinib alone could inhibit the proliferation of H526 cells in a dose dependent manner, and its IC50 value was 47.3 μM (**Figure 3B**). The data also demonstrated that crizotinib alone could also inhibit the proliferation of H526 cells in a dose dependent manner, and its IC50 value was 16.7 μM (**Figure 3D**). Moreover, the combination treatment, which consisted of imatinib and crizotinib, had more significant inhibitory effects on the proliferation of H526 cells than 47.3 μM imatinib alone or 16.7 μM crizotinib alone ($P < 0.001$, **Figure 4**).

Effects of the combination of imatinib and crizotinib on SCLC cells in vivo

To investigate whether the combination of imatinib and crizotinib was a potential therapy targeting c-Kit and c-Met for SCLC cells, an animal model was used. It was observed that the tumor tissues from mice treated with imatinib

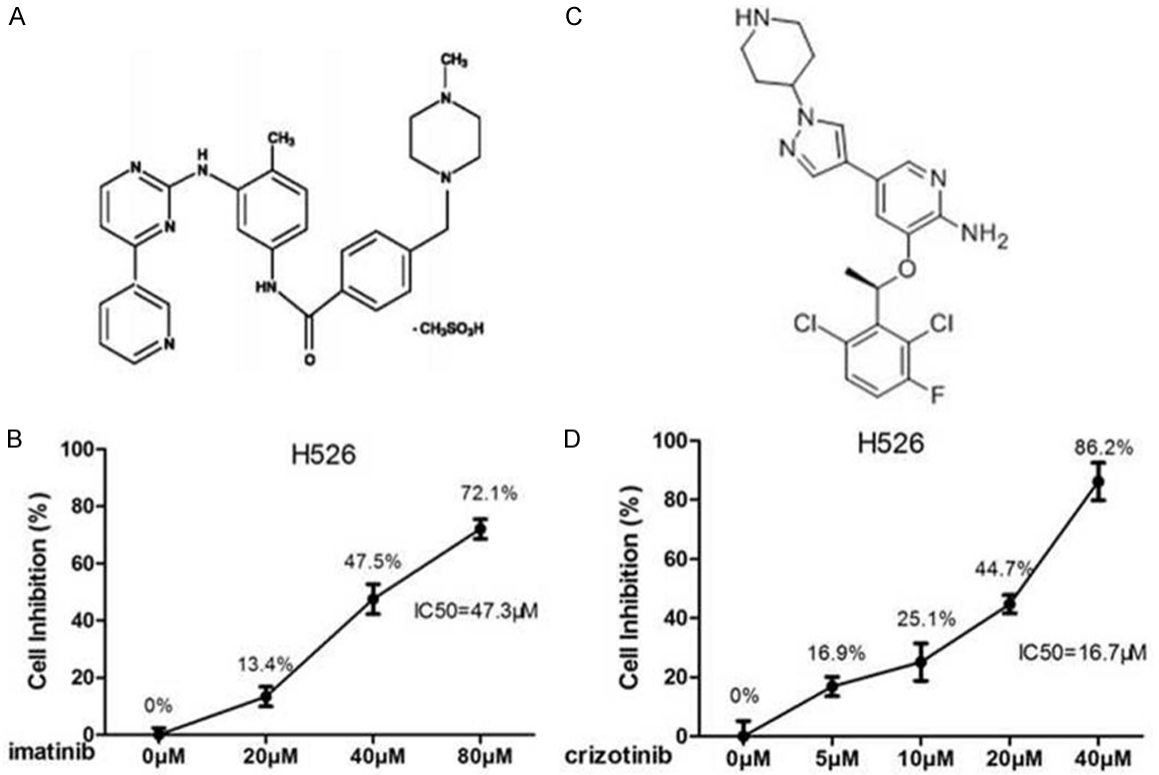

Figure 3. The effects of imatinib and crizotinib on cell proliferation in H526 cells. A. The structure of imatinib. B. The effect of imatinib on cell proliferation in H526 cells measured with CCK-8 assay after treating the cells with different concentrations of imatinib for 24 h. C. The structure of crizotinib. D. The effect of crizotinib on cell proliferation in H526 cells measured with CCK-8 assay after treating the cells with different concentrations of crizotinib for 24 h. IC50 values of imatinib and crizotinib for H526 cells were measured with the data of the inhibitory effects under different drug concentrations. The data represent three independent experiments and are presented as the mean ± SD.

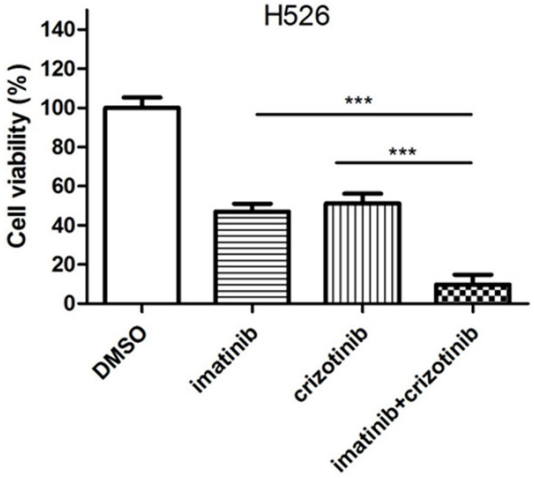

Figure 4. Effects of imatinib alone, crizotinib alone and their combination on the cell proliferation of H526 cells. The cell proliferation of H526 cells was measured with CCK-8 assay after the cells were respectively treated with 47.3 μM imatinb alone, 16.7 μM crizotinib alone and their combination for 24 h. The data represent three independent experiments and are presented as mean ± SD. The data were then analyzed by the LSD method (Least significant method) in a one-way ANOVA analysis. ***P < 0.001.

and crizotinib were smaller than those from mice treated with imatinib alone or crizotinib alone or from saline after injection with H526 cells (**Figure 5A**). The data demonstrated that the combination of imatinib and crizotinib had a more significant ability to decrease the size of tumors induced by H526 cells than either imatinib alone or crizotinib alone.

It has been reported that imatinib could inhibit the activity of c-Kit and that crizotinib acts as an inhibitor of ALK, c-Met and RON [20-22]. It was found in this study that c-Kit and c-Met are highly expressed in H526. Animal tests were used to investigate whether the inhibitory effects of imatinib alone, crizotinib alone, or their combination was more significant against SCLC with higher expression levels of c-Met and c-Kit.

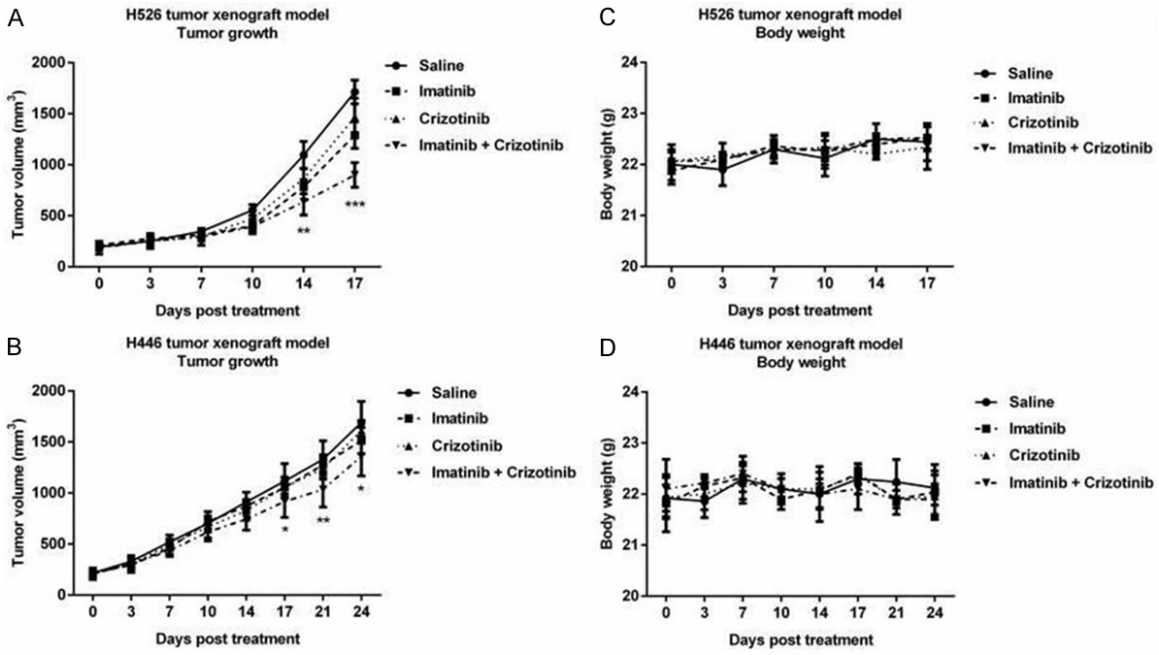

Figure 5. The effects of the combination of imatinib and crizotinib on tumor volume and body weight induced by SCLC cells in vivo. A. The inhibitory effects of imatinib alone, crizotinib alone and their combination on the tumor volume of the tumors induced by H526 cells. B. The inhibitory effects of imatinib alone, crizotinib alone and their combination on tumor volume of the tumors induced by H446 cells. C. The effects of imatinib alone, crizotinib alone and their combination on the body weight of the mice inoculated with H526 cells. D. The effects of imatinib alone, crizotinib alone and their combination on the body weight of the mice inoculated with H446 cells. The data are presented as mean ± SD (n = 5) and compared by the LSD method (Least significant method) in two-way ANOVA analysis. *P < 0.05, **P < 0.01, ***P < 0.001 compared with Imatinib alone or Crizotinib alone group.

It was observed that the inhibitory effects of imatinib alone, crizotinib alone, or their combination on tumor volume were more significant for the tumors induced by H526 cells than those induced by H446 cells (**Figure 5A**, **5B**). However, the effects of imatinib alone, crizotinib alone, or their combination on body weight had no apparent difference between the mice injected with H526 cells and the mice injected with H446 cells (**Figure 5C**, **5D**).

Effects of the combination of imatinib and crizotinib on p-c-Kit and p-c-Met

c-Kit and c-Met play important roles in cell proliferation, especially in cancer cells, such as NSCLC [9, 16]. Moreover, c-Kit and c-Met can be activated by phosphorylation [20-22]. It is thought that Imatinib and crizotinib have a great inhibition on the activity of c-Kit and c-Met by decreasing their phosphorylation levels. To test the possibility that the inhibition of imatinib and crizotinib on the tumor volumes of the tumors induced by H526 cells resulted from the diminishing activity of c-Kit and c-Met, in other words a decrease of the phosphorylation of c-Kit and c-Met, the tumor tissues from the tumors induced by H526 cells were sliced into thin sections and incubated with primary antibodies against p-c-Kit and p-c-Met. Data indicated that the combination could more significantly inhibit the phosphorylation of c-Kit and c-Met than imatinib alone or crizotinib alone (**Figure 6**).

Discussion

SCLC, which is a malignant and aggressive tumor with low 5 year survival rates (less than 5%), differentiates from non-small cell lung cancer because of its shorter doubling time and more rapid metastasis. Although SCLC shows sensitivity to chemotherapy and radiotherapy, most patients may die from recurrence and metastasis. However, the mechanisms of recurrence and metastasis in SCLC are still not fully understood. Therefore, a better understanding of the molecular mechanisms about SCLC would probably contribute to determining novel drug targets and developing the new therapies.

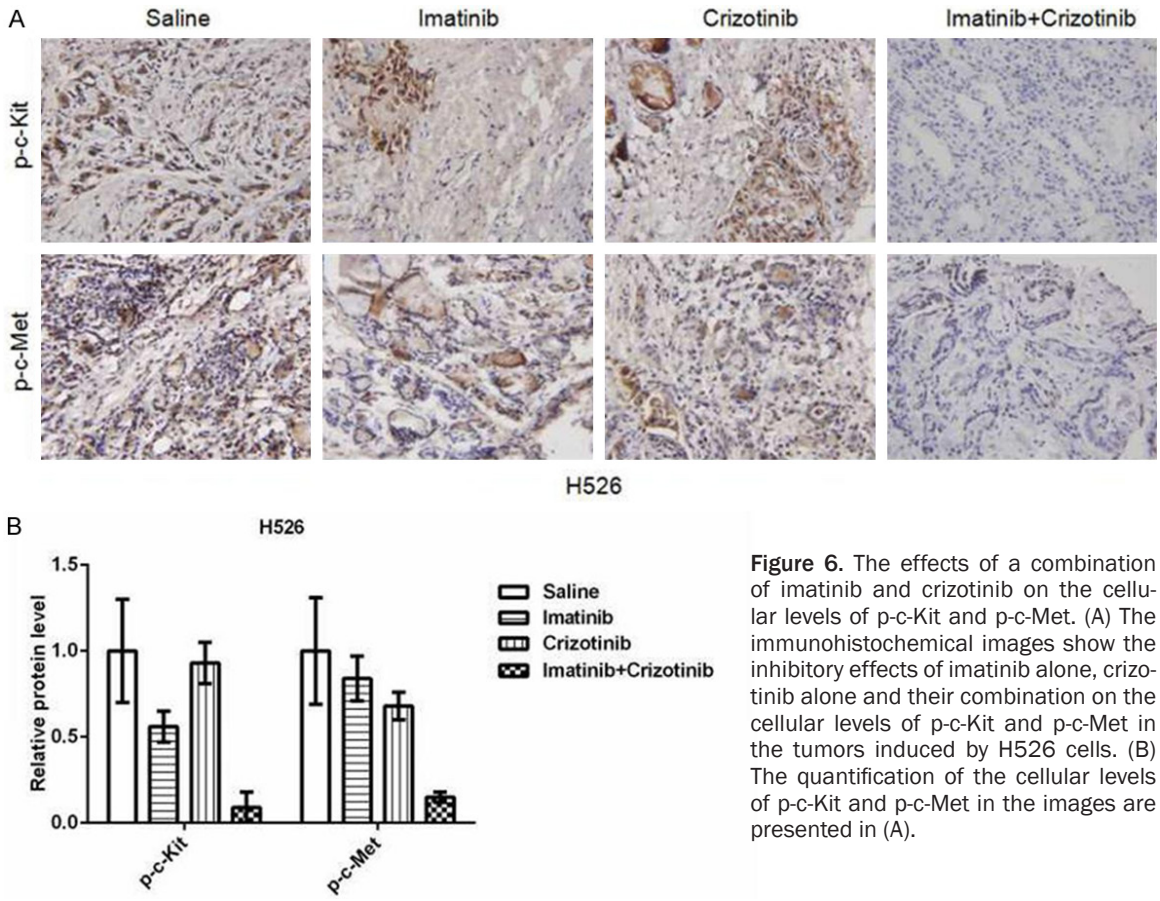

Figure 6. The effects of a combination of imatinib and crizotinib on the cellular levels of p-c-Kit and p-c-Met. (A) The immunohistochemical images show the inhibitory effects of imatinib alone, crizotinib alone and their combination on the cellular levels of p-c-Kit and p-c-Met in the tumors induced by H526 cells. (B) The quantification of the cellular levels of p-c-Kit and p-c-Met in the images are presented in (A).

Receptor tyrosine kinases have been identified as potential targets for cancer therapy. With the development of cancer targeting therapies, one of the most important challenges is how to design specific strategies to target the crucial signaling pathways involved in different tumors. There are plenty of key regulators, which control cell function by regulating signaling pathways, such as c-Kit and c-Met. These two factors play important roles in cell proliferation, which have been reported to be expressing in numerous carcinomas [8, 9, 16]. c-Kit has the ability to activate signaling pathways involved in cell proliferation after being combined by its ligand SCF [14]. The HGF/c-Met signaling pathway contributes to cell proliferation by activating Akt and other relevant factors [7, 24].

Previous studies have identified the expression of either c-Met or c-Kit in some SCLC cell lines [25], but few publications have reported on the expression of these two in the same cell lines simultaneously. In this study, to investigate the correlation of the expression of c-Kit and c-Met with cell proliferation, the content of c-Kit and c-Met and cell proliferation in diverse SCLC cell lines was detected. It was found that there was a positive correlation between cell proliferation and the expression of c-Kit and c-Met in SCLC cells. This pointed to the possibility of using c-Kit and c-Met as the treatment targets.

In this study, H526 cells showed different toxic responses to imatinib and crizotinib in a dose dependent manner, and the IC50 values were 47.3 μM for imatinib and 16.7 μM for crizotinib (**Figure 3**). Although neither imatinib nor crizotinib had an anti-proliferation effect in H526, we aimed to improve the inhibitory effect by targeting both c-Met and c-Kit, so then the combination of imatinib and crizotinib was used, and it was exciting that the combination exhibited more significant therapeutic effects than either drug alone with IC50 concentration on H526 ($P < 0.01$, **Figure 4**).

Animal tests presented in **Figure 5** demonstrated that the combination of imatinib and crizotinib had a more significant ability to decrease the size of tumors induced by H526 cells than

imatinib alone or crizotinib alone, and the inhibitory effects of imatinib alone, crizotinib alone or their combination on tumor volume were more significant for the tumors induced by H526 cells than those induced by H446 cells. Because H526 cells had high expression levels of c-Met and c-Kit than H446 cells, the above in vivo results confirmed the possibility of using c-Kit and c-Met as the treatment targets. The immunohistochemistry tests in **Figure 6** clarified the possible mechanism of the use of c-Kit and c-Met as the treatment targets, which enriched this study.

To summarize, this study made the following important conclusions: (1) c-Kit and c-Met might be used as treatment targets for SCLC; (2) the combined treatment with both imatinib and crizotinib might be more effective in treating SCLC than imatinib alone or crizotinib alone; (3) the mechanisms for imatinib alone, crizotinib alone or their combination to treat SCLC might include the inhibition of the phosphorylation of c-Met and c-Kit.

Acknowledgements

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Disclosure of conflict of interest

None.

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