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Gleevec (STI-571) inhibits lung cancer cell growth (A549) and potentiates the cisplatin effect in vitro

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Abstract

Background: Gleevec (aka STI571, Imatinib) is a recently FDA approved anti-tumor drug for chronic myelogenous leukemia. Gleevec binds specifically to BCR-ABL tyrosine kinase and inhibit the tyrosine kinase activity. It cross-reacts with another two important membrane tyrosine kinase receptors, c-kit and PDGF receptors. We sought to investigate if Gleevec has a potential role in treatment of non-small cell lung cancer.

Results: We have shown that Gleevec alone can inhibit the A549 lung cancer cell growth in dose-dependent manner, and the optimal concentration of Gleevec inhibition of A549 cell growth is at the range of 2–3 μM (IC50). We have also shown that A549 cells are resistant to cisplatin treatment (IC50 64 μM). Addition of Gleevec to the A549 cells treated with cisplatin resulted in a synergistic cell killing effect, suggesting that Gleevec can potentiate the effect of cisplatin on A549 cells. We also showed that the A549 lung cancer cells expresses the platelet derived growth factor receptor α, and the inhibitory effects of Gleevec on A549 cells is likely mediated through inhibition of PDGFR α phosphorylation. We further tested 33 lung cancer patients' tumor specimens to see the frequency of PDGFR-α expression by tissue micro-arrays and immunohistochemistry. We found that 16 of the 18 squamous carcinomas (89%), 11 of the 11 adenocarcinomas (100%), and 4 of the 4 small cell lung cancers (100%) expressed PDGFR-α.

Conclusion: These results suggest a potential role of Gleevec as adjuvant therapeutic agent for treatment of non-small cell lung cancer.

Background

Lung cancer is the leading killer of all cancer patients. It is generally classified as small cell carcinoma and non-small cell carcinoma. Treatment of lung cancer is less than optimal and the mean survival for advanced lung cancer patient is less than one year regardless what treatment regimen was used [1]. A new approach other than conventional chemoradiation therapy is needed for prolonged survival of lung cancer. Emerging new treatment modalities are generally targeted to specific tyrosine kinases of the tumor cells through basically two independent approaches [2]. One is to use highly specific monoclonal antibody to target the membrane receptors of growth factors important for tumor cell growth, and the antibody/antigen complex evokes host immune system to kill the tumor cells. This approach is exemplified by Her2/neu receptor

in breast cancer patients and Herceptin [3]. The second approach is to develop small organic molecules targeting the specific tyrosine kinases in the signaling pathway in the tumor cells that can easily gain access into the tumor cells. This approach is best exemplified by Gleevec and BCR-ABL fusion kinase in chronic myelogenous leukemia [4].

Gleevec (also known as STI571, Imatinib from Novartis Pharmaceutical Inc.), a recently FDA approved drug for chronic myelogenous leukemia, is an ATP analogue, and it competitively binds to and inhibits BCR-ABL tyrosine kinase, resulted from the chromosomal translocation t(9; 22). Gleevec has been shown to induce clinical, hematological and molecular remissions for CML patients [4]. It cross-reacts with two other important growth factor receptors containing tyrosine kinase domains, c-kit and PDGF receptors, that play important roles in growth and proliferation of a variety of cell types [5]. C-kit (also known as CD117) is frequently mutated in gastrointestinal stromal tumor (GIST), and the mutated c-kit shows higher tyrosine kinase activity. Gleevec has been demonstrated to inhibit the growth and proliferation of GIST, and induce complete or partial clinical remission in GIST patients [6,7]. Recently Gleevec was showed to inhibit the growth of dermatofibrosarcma protuberans (DFSP) through inhibiting the PDGF receptor [8,9].

As a part of effort to evaluate the newly emerging drugs for lung cancer, we studied the role of Gleevec on non-small cell lung cancer. We previously showed that non-small cell lung cancers express minimal (negligible) level of c-kit (Zhang, P., unpublished). Therefore it is unlikely that Gleevec exerts the effect, if any, through c-kit on non-small cell lung cancer. We reported here a study of inhibitory effect of Gleevec on lung cancer cells (A549 cells) in vitro. We showed that Gleevec alone can inhibit the growth and proliferation of A549 cells at the known therapeutic concentration for CML. Addition of Gleevec to A549 cells with cisplatin induced cell death synergistically, suggesting Gleevec can potentiate the cisplatin effect on A549 cells. We have demonstrated that A549 cells express PDGFR α, one of the known potential targets for Gleevec effect. The inhibitory effect of Gleevec on the A549 cells is likely mediated through inhibition of PDGF receptor α phosphorylation. We further tested 33 lung cancer patients' tumor specimens, and showed that most of the lung cancer tumor specimens expressed PDGFR-α. These results provided important in vitro data to support the notion that Gleevec can inhibit the A549 cell growth and proliferation, and may potentially offer a treatment option for lung cancer either alone or in conjunction with chemotherapy drug cisplatin.

Results

Gleevec inhibited the A549 cell growth in a dose-dependent manner

We first test to see if Gleevec can inhibit the growth of A549 cells under the culture conditions. We have chosen to perform the experiments under the normal culture condition since we reasoned that the effect of Gleevec on tumor cells, if any, should be under the normal, not serum free condition. Therefore, we chose to add the drug directly into the culture medium containing 5% fetal calf serum. A549 lung cancer cells were plated at two cell densities into the 96 well plates, and Gleevec was added to the culture medium. We used the MTT assays to assess the viability of the tumor cells treated with or without the drug. It was evident that increasing concentrations of Gleevec in the culture medium inhibited the growth of A549 cells in a dose dependent manner. The concentration of Gleevec to inhibit 50% cell growth (IC50) was estimated to be around 2–3 μM (Figure 1), whereas the IC50 of cisplatin on A549 cells was estimated to be 64–70 μM (Figure 2). We have also seen that Gleevec can inhibit the growth of human 293 kidney cells, but at slightly higher concentration (4 μM). The IC50 concentration for A549 cells didn't appear to have inhibitory effects on human 293 cells (Figure 3). These results showed that Gleevec alone can indeed inhibit the growth of A549 lung carcinoma cells. The inhibitory effect of Gleevec on the A549 cells appeared to be in a therapeutic range of the drug as demonstrated in chronic myelogenous leukemia cells from the patients.

Gleevec potentiated the cell-killing effect of cisplatin

One of the major reasons of testing Gleevec for treatment of non-small cell lung carcinoma is to see if the specific tyrosine kinase inhibitor Gleevec, and other potential small molecules currently on clinical trials, can be used as an adjuvant therapeutic agent in combination with conventional chemotherapy. We sought to see if Gleevec can potentiate the cell killing effects of cisplatin, the leading chemotherapeutic drug for almost all advanced cancer patients. We used the IC50 concentrations for both Gleevec and cisplatin to treat A549 cells in culture, and assessed the viability of the cells by MTT assays at the different time points (Figure 4). Gleevec (2 μM) and cisplatin (64 μM) both inhibited the growth of the tumor cells individually after treatment of the cells for 48 hour. Combination of Gleevec and cisplatin synergistically inhibited the growth of A549 cells (Figure 4). This synergistic effect of Gleevec and cisplatin on A549 lung cancer cells were not seen for human 293 kidney cells (Figure 5).

Expression of PDGFR-α in the A549 lung cancer cells

Since the effect of Gleevec is mediated through inhibiting BCR-ABL tyrosine kinase, c-kit, and PDGF receptors, we are interested in the potential target for Gleevec effect in

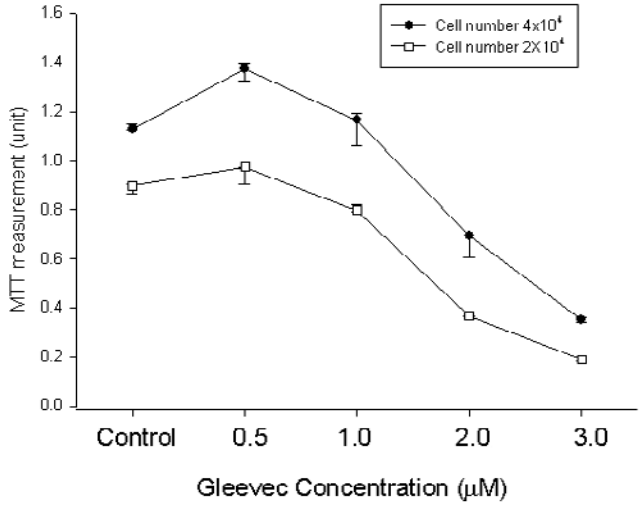

Figure 1
Inhibitory effects of Gleevec on A549 lung cancer cells in a dose-dependent manner using two different cell densities in the serum-containing medium. Gleevec concentrations were as listed ranging from 0.5 to 8 μM. Unless stated otherwise, the cell culture was maintained and treated with the drugs for 48 hours before being analyzed by MTT assays. The results were the mean of five different readings (± 2SD) within in one experiment, and the figures were representative of three independent experiments.

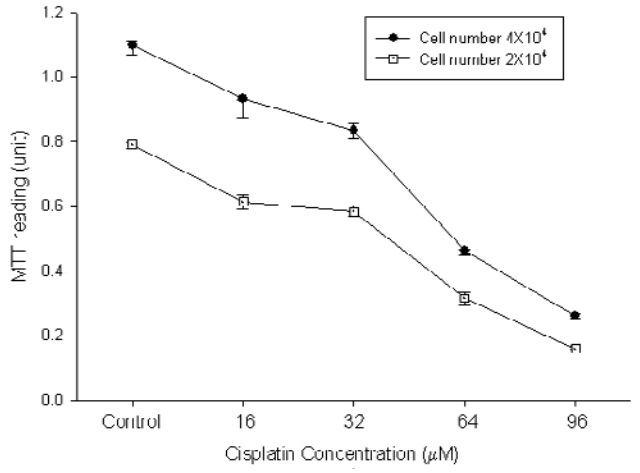

Figure 2
Dose-dependent inhibition of A549 cell growth by cisplatin using two different cell densities. Cisplatin concentrations were used as listed ranging from 16 to 96 μM.

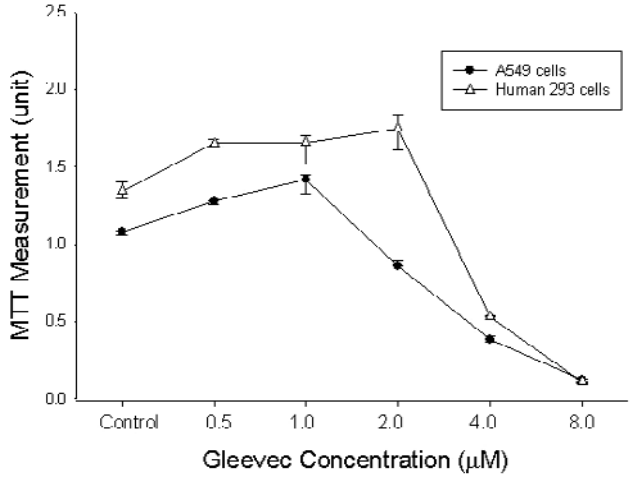

Figure 3
Comparison of Gleevec effects on A540 cells with the human 293 kidney cells. A549 cells and human 293 cells were cultured and treated with Gleevec as described above using the concentrations listed. The culture conditions were identical to those described in Figure 1.

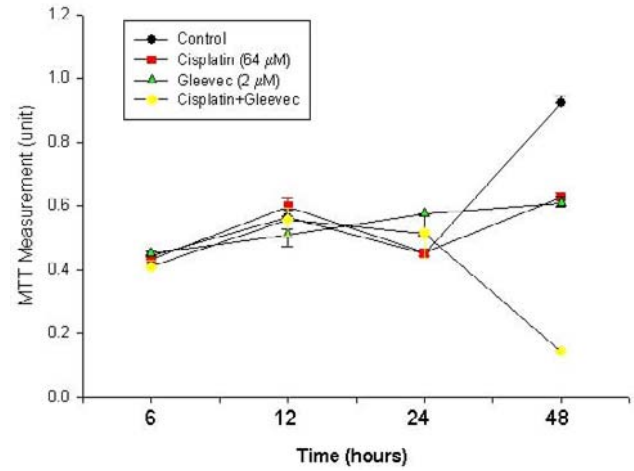

Figure 4
The effects of either Gleevec or cisplatin alone or in combination on A549 lung cancer cells. The tumor cells were treated with either drug alone or in combination for various times using IC50 concentrations for both Gleevec (2 μM) and cisplatin (64 μM). The synergistic effect was seen at 48 hours after treatment. Control cells were treated with neither drug.

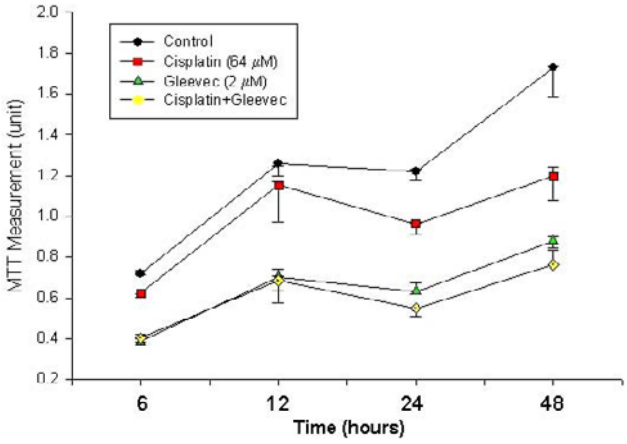

Figure 5
The effects of either Gleevec or cisplatin alone or in combination on human 293 kidney cells. The drug concentrations were as described for Figure 4. Control cells were treated with neither drug.

the lung cancer cells. We have previously showed by immunohistochemical staining that in 54 primary non-small cell lung carcinoma specimens, expression of c-kit was weak and minimal, if any (Tang and Zhang, unpublished observation). We sought to determine the expression levels of PDGF receptors α and β in A549 cells and the primary non-small cell lung carcinoma by immunofluorescent and immunohistochemical staining methods. In A549 cells, expression of PDGFR-α, not PDGFR-β, was detected with strong cytoplasmic and membrane staining patterns (Figure 6 and 7). There was no demonstrable nuclear staining signal. We further detected the presence of PDGFR-α in the whole cell lysates of A549 cells by Western blotting analysis (Figure 8). A549 cells were cultured under the condition described, and the cells were lysed and the whole cell proteins were separated on 7.5% SDS-PAGE. The proteins were transferred onto nitrocellulose membrane, and the PDGFR-α was detected with anti-PDGFR-α antibody (Santa Cruz Biotechnologies Inc.). Treatment of the cells with Gleevec at two concentrations did not influence the expression of PDGFR-α expression in the cells. PDGFR-β was not detected in A549 cells using both immunofluorescent staining and Western blot analyses (Data not shown).

Microarrays and immunohistochemical staining

Existing data suggest that Gleevec can exert its inhibitory effect on tumor cells through inhibiting PDGF receptor function, and we have shown that the primary non-small cells lung cancer expressed minimal c-Kit. We would like to see if PDGF receptor expression in lung cancer patients is frequent finding, and this could potentially provide a cellular mechanism by which Gleevec inhibits the lung cancer tumor growth. We used the tumor tissue micro-array technique using the high-density lung tissue arrays from Clinomic Biosciences Inc, MA. Lung tissue micro-array slides containing 33 primary lung cancer tumor specimens with the corresponding normal lung tissues were used for immunohistochemical staining using anti-PDGFR-α and β antibodies as described. The clinical diagnoses of lung cancers were reviewed and confirmed. Using clinical diagnostic criteria, the immunostaining patterns and intensities were verified by two independent board certified surgical pathologists. Since immunohistochemical staining is a semi-quantitative method, we scored the staining intensities arbitrarily as 1+, 2+ and 3+ (Figure 9). There were 18 cases of squamous carcinomas of the lung within the tissue array slide, and 16 of the 18 cases were positive for PDGFR-α (89%). 7 of the 16 cases were scored 1+ (39%), 5 cases 2+ (28%) and 4 cases 3+ positivity (22%) (Table 1). There were 11 of the 11 adenocarcinoma specimens that were positive for PDGFR-α (100%) [6 cases 1+ (55%), 3 cases 2+ (27%), 2 cases 3+ (18%) respectively]. Small cell carcinomas were also positive expression of PDGFR-α [4 cases 2+ (100%)]. Two cases of malignant mesotheliomas were negative for PDGFR-α. The corresponding normal lung tissues were found to be negative for PDGFR-α. Although there were some interstitial staining signals for PDGFR-β, the tumor tissues were negative for PDGFR-β (Figure 7, micro-array data not shown). These results demonstrated that PDGFR-α expression is elevated in the tumor tissues compared to normal lung parenchyma, and the expression of PDGFR-α in tumor tissue may play important roles in tumor growth and progression.

Discussion

Gleevec is the first tyrosine kinase inhibitor that has been proven to be effective for clinical cancer patients, and its design is based purely on the inhibitory effect of the compound on the intracellular BCR-ABL tyrosine kinase. This represents a new direction in drug design targeting specific tyrosine kinases important for intracellular signal transduction pathways. There are many other small organic molecules targeting different tyrosine kinases in horizon. From the clinical standpoint, it will be of great importance to see this line of new molecules to synergize with conventional chemotherapy or sensitize the tumor cells to conventional chemoradiation therapy. Our current study showed that the small organic molecules such as Gleevec targeting the specific tyrosine kinases can not only inhibit the tumor cell growth alone, but also synergize with cisplatin in induction of tumor cell death.

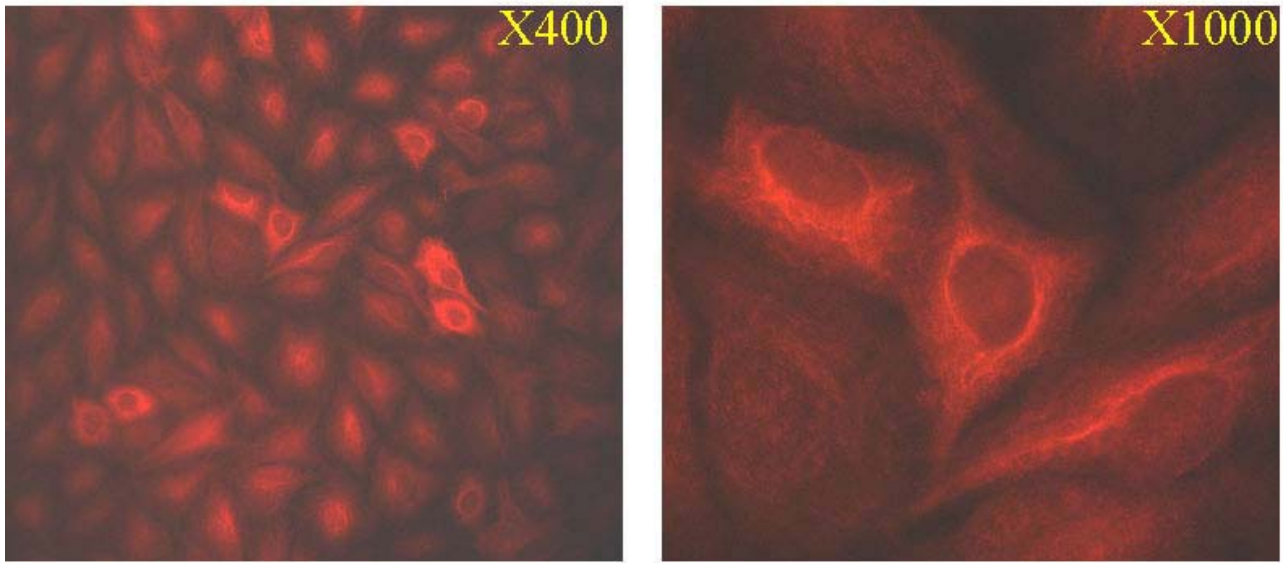

Figure 6
Immunofluorescent stainings of A549 cells using anti-PDGFR-α and β antibodies. The secondary antibody was conjugated with Texas red fluorescent dye. The photographs were taken at the magnifications of 400X and 1000X.

Gleevec effects vs drug toxicity

We have shown in this study that Gleevec alone can inhibit the growth of A549 cells at the concentration of 2–3 μM (IC50). This is within the known therapeutic ranges for patients with CML, since the plasma levels of Gleevec inducing hematologic and cytogenetic response in patients with CML were reported to be in the range of 0.1–3.4 μg/ml (0.17–5.68 μM) after treatment with 25–600 mg /day [4]. In our system, the level of Gleevec at the tested concentration was in the low micromolar ranges, and the inhibitory effect of Gleevec on A549 cells is likely to be genuine through binding to the receptor, not due to the toxic effects of the drug. This inhibitory effect can also be seen with the human kidney 293 cells with slight higher IC50. At the concentration of 4 μM or higher, the inhibitory effect of Gleevec on both A549 and 293 cells were found to be identical (Figure 2 and 3). Higher concentration than 6 μM in the cell culture condition is unlikely to be translated to clinical patients, since the adverse effects of the drug will become intolerable for the patients.

Potential target of Gleevec in lung cancers

Gleevec was designed to inhibit the BCR-ABL tyrosine kinase, and it cross-reacts with c-kit and PDGFR [5]. In non-small cell lung cancer, BCR-ABL expression has never been reported, and it is unlikely that BCR-ABL fusion protein plays any role in lung cancer. There has been no report to link cellular ABL kinase mutation to any cancer patients except BCR-ABL in CML, although the detailed c-Abl function has not been extensively studied in lung cancer patients. CAbl is a tyrosine kinase that plays important roles in cell growth, differentiation and apoptosis. Although c-Abl mutations have not been reported in human cancers, c-Abl related protein, ARG (or Abl-2), was shown to be regulated in non-small cell lung cancers by hypermethylation (Dr. Steven Reynolds, National Institute of Occupational and Safety Health, Morgantown, WV, personal communication). ARG shares significant homology with c-Abl throughout the amino acid sequences. Gleevec has been shown to inhibit ARG tyrosine kinase [10](Dr. Jean Wang, Biology, University of California San Diego, personal communication), although the functional significance of ARG kinase in lung cancer is yet to be elucidated.

We have studied the expression of c-kit (CD117) in the non-small cell lung cancer patients, and we found that approximately 13% of the tumor specimens were weakly positive for c-kit. The level of expression is generally weak (1+). These results lead us to find an alternative target for Gleevec, because of the low expression level and low frequency of expression of c-kit in the lung cancer patients.

PDGFR expression in lung cancer

We have shown convincingly that lung cancer cell A549 expressed PDGFR-α both by immunostaining the cells and by Western blot analysis. PDGF receptors are widely

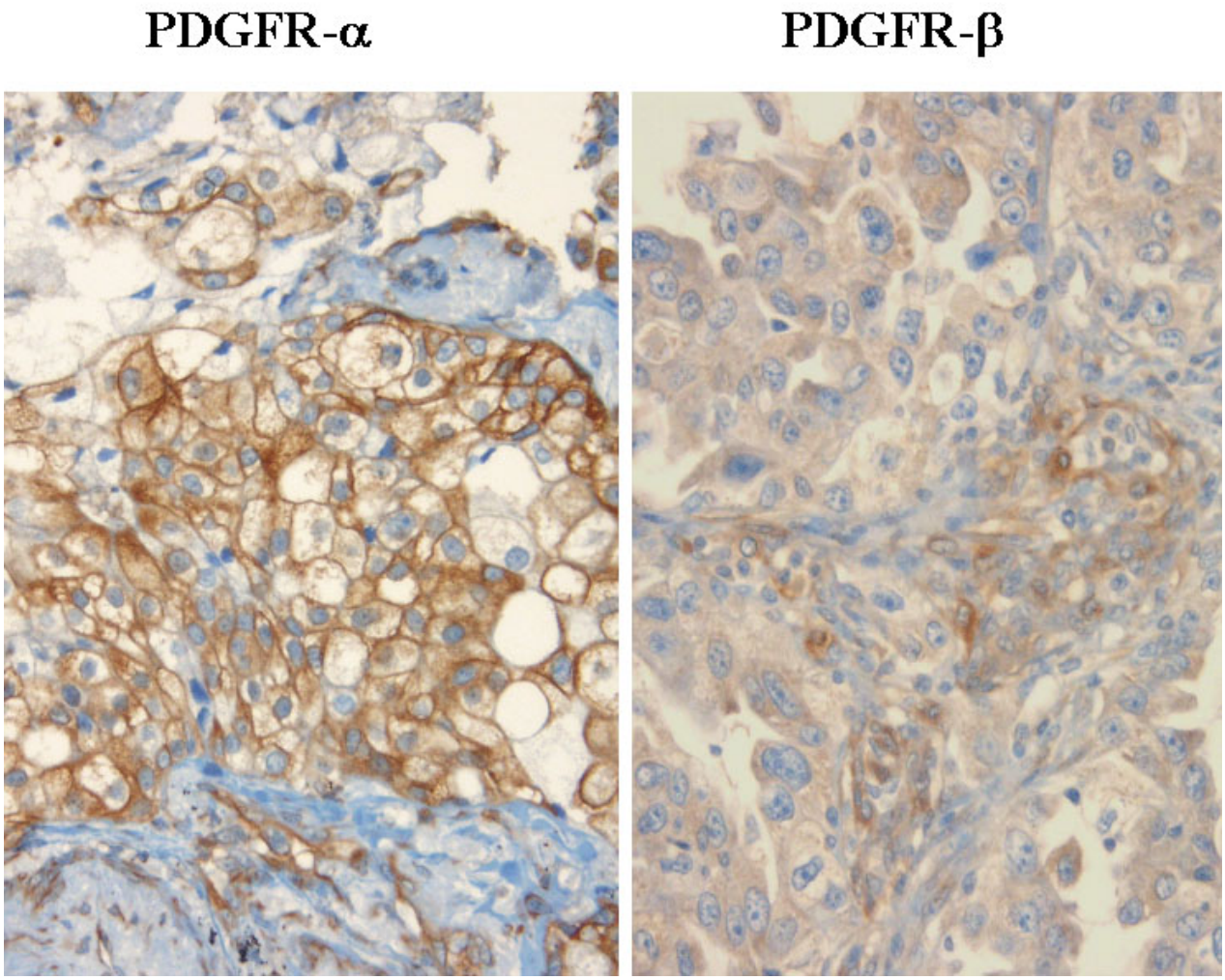

Figure 7
Immunohistochemical staining of human non-small cell lung cancer tumor specimen with anti-PDGFR-α antibody. The photographs were taken at the magnifications of 400X.

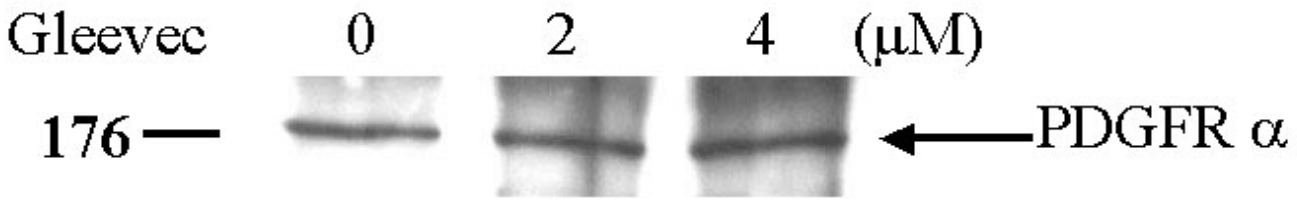

Figure 8
Western blot analyses of PDGFR-α in the whole A549 cell lysates. The whole cell lysates were separated on 7.5% SDS-PAGE and analyzed by Western blot using anti-PDGFR-α antibody. Treatment of A549 cells with Gleevec showed no effect on the PDGFR-α protein expression.

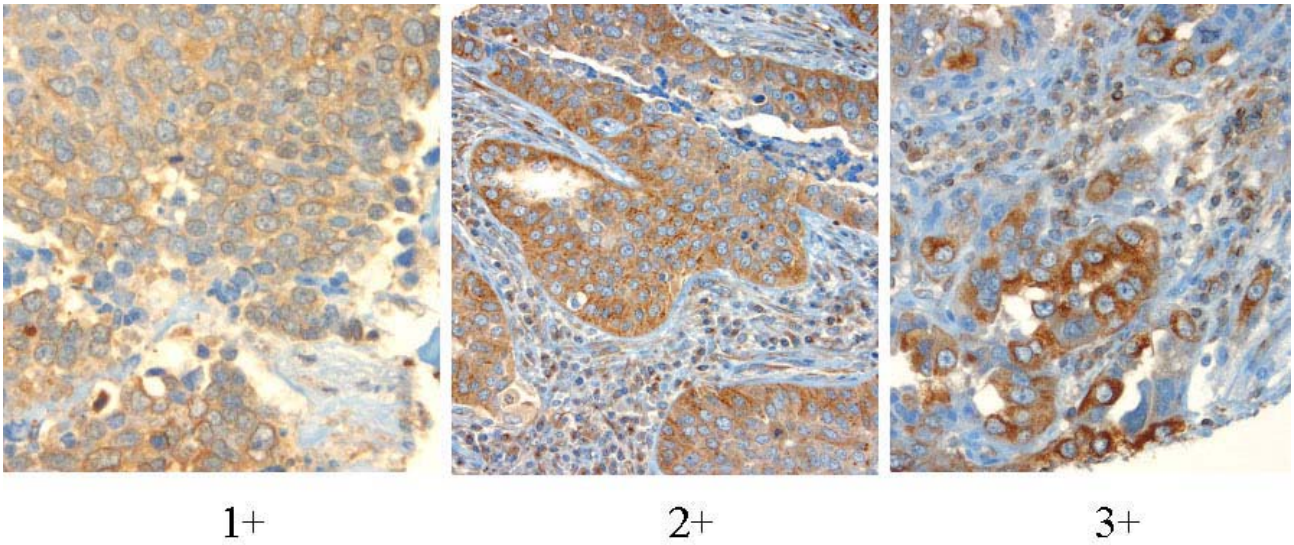

Figure 9
Immunohistochemical staining of lung cancer tumor specimens using the tissue micro-arrays and the anti-PDGFR-α antibody. The staining intensities were scored 1+, 2+ and 3+ for all the tumors. The staining patterns were cytoplasmic and membrane. The magnification was at 400X for all the sections.

Table 1: Summary of PDFGR-α expression in lung cancer specimens

Cancer type	Total	Negative	Positive			% positive
			1+	2+	3+	
Squamous carcinoma	18	2	7 (39%)	5 (28%)	4 (22%)	89%
Adenocarcinoma	11	0	6 (55%)	3 (27%)	2 (18%)	100%
Small cell carcinoma	4	0	0	4 (100%)	0	100%
Mesothelioma	2	2				0%

expressed in a variety of tissues, and the levels of expression in normal tissues are minimal. Over-expression of PDGFR has been reported in a variety of human tumors including glioma and glioblastoma, pancreatic and colonic cancers, breast, bone and ovarian tumors [11]. In normal cells, expression of PDGFR can be seen in the fibroblasts and the smooth muscle cells in the lung and airway [12]. There is so far to our knowledge no report to link the expression of PDGFR to lung cancer. Since it has been reported that Gleevec can inhibit the function of PDGFR under the cell culture conditions, it is likely that Gleevec exerts its inhibitory effects on A549 lung cancer cells through inhibiting the PDGFR function.

Synergistic effect of Gleevec and cisplatin on A549 cells

We have shown that Gleevec can exert its effect synergistically with cisplatin. Cisplatin causes two types of DNA damage, DNA adducts and inter-stranded cross-linking, resulting in activation of apoptosis pathway in the target tumor cells [13,14]. Our results suggest that inhibition of tyrosine kinase activity of the potential targets by Gleevec appear to potentiate the effect of cisplatin in DNA-damage induced apoptosis in A549 cells. Although the underlying mechanism of such synergism is unclear, it is of great interest to combine the two drugs in clinical lung cancer patients to see if the synergy of the two drugs exists under the physiological conditions. It has been widely thought, but never been proven that the specific tyrosine kinase inhibitors such as Gleevec can be used as adjuvant therapy in combination with conventional chemotherapy, because the limited clinical trial data suggests only a marginal benefits using this kind of drug to treat the cancer alone (John Rogers, WVU Cancer center, ECOG trial member, personal communication). Current study results suggests that

Gleevec can potentiate the cisplatin effect on A549 lung cancer cells, and these findings provide important in vitro data for further testing the possibility of using Gleevec as adjuvant therapy for clinical lung cancer patients.

Methods

Cell culture and drug testing

Lung cancer cell line A549 cells were cultured in DMEM with 5% fetal calf serum as described. The cells are seeded in 96-well plate at the cell density of 2–4 × 10^4 per well as indicated, and the tumor cells were cultured in the medium containing serum. Cisplatin was purchased from Sigma Chemicals (St. Louis, MO), and dissolved in water at the concentration of 32 mM. The Gleevec (Novartis Pharmaceuticals. Inc., NJ) was purchased from the outpatient pharmacy at West Virginia University and dissolved in water at the concentration of 1 mM. The experimental procedure for the cisplatin and Gleevec treatment was the following: The A549 cells and human 293 cells were plated into the 96 well plates at the cell density indicated and the cells were allowed to attach overnight. The attached cells in the plates were washed once with PBS and replaced with fresh medium containing various concentrations of drugs indicated. The MTT assays were performed after 48 hours of continuous incubation with medium containing the drugs. The values shown in the figures are mean readings from five wells in each experiments and representative of three independent experiments.

MTT assays

We have performed a series of MTT assays to determine the effects of the anti-tumor drug STI571 on the number of A540 lung cancer cells and human 293 kidney cells. The assay is based upon the cleavage of the yellow tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] to purple formazan crystals by metabolically active cells [15]. The tested concentration of Gleevec ranged from 0.5 to 8 μM. The tumor cells were seeded into 96-well culture plate, and maintained for culture for 24 hours before the first compound was added to the medium. The cultured cells were incubated in a medium containing 5% serum and treated with Gleevec for 48 hours. After treatment, 10 μl of MTT labeling reagent were added to each well and plates were incubated at 37°C for 4 h. Following MTT incubation, the cultures were solublized and the spectrophotometric absorbance of the samples was detected by using a microtiter plate reader. The wavelength to measure absorbance of formazan product is 570 nm, with a reference wavelength of 750 nm.

Immunofluorescent staining of the cultured cells

The A549 cells were cultured as described on the coverslips, and fixed with 3.7% paraformaldehyde for 10 minutes at room temperature. The fixed cells were permeablized with 3% Triton X-100 in PBS and directly used for immunofluorescent and immunocytochemical stainings. The primary anti-PDGFR-α antibody was purchased from Santa Cruz Biotechnologies Inc (Santa Cruz, CA). The secondary antibody for immunofluorescent labeling was from Molecular Probe Inc. (Portland, OR).

Lung tumor tissue microarrays and immunohistochemical staining

The high-density lung cancer tissue microarray slides were made in Clinomics Bisciences Inc. MA, and was used for immunohistochemical staining for PDGFR-a. Briefly, the tissue microarray slides were sectioned at 5 micron in thickness and heated to 65°C for 10 minutes to de-parafinize the tissues. The slides were washed three times in xylene, then dehydrated and rehydrated in 100%, 95% and 70% ethanol. The tissue sections were finally washed with PBS once before going through the antigen retrieval process. The antigen retrieval was performed using citrate buffer (pH 7.4) at 90°C for 30 minutes. The immunohistochemical staining was performed using Ventana Bench Mark II automated staining device following the manufacturer's instruction (Ventana Medical Inc. Tucson, AZ). The primary PDGFR-α antibody was from Santa Cruz Biotechnologies Inc (Santa Cruz, CA). The tumor section slides and the immunostaining patterns were reviewed by two independent practicing surgical pathologists, and the signal intensities were scored at 1+, 2+ and 3+. The staining characteristics of all tumors were summarized in Table 1.

Western blotting

The A549 cells were maintained in the culture condition as described above. Western blotting analyses of PDGF receptors were performed using a previously described method [16]. Briefly, the tumor cells were treated with Gleevec at the various concentrations for 6 hours. The whole cell extracts were prepared and used for Western blot with anti-PDGF receptor α using RIPA buffer containing SDS. The whole cell extracts were separated on the 7.5% SDS PAGE and transferred onto nitrocellulose membrane by electroblotting. The primary antibody was incubated in the 5% non-fat milk with the proteins on the membrane overnight at 4°C, and the protein of interest was visualized by the enhanced chemiluminescent method (ECL). The anti-PDGF receptor α antibody was used at 1:500 (Santa Cruz Biotechnologies Inc. CA).

Author's contributions

PZ designed, organized the whole study and analyzed all the data. WG performed a majority of the experiments in the study. ST provided micro-array slides for lung cancers, and discussed extensively regarding the design and execution of the study. BSD reviewed the patient's tumor specimens. All authors read and approved the final manuscript.

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