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The Role of IGFBP7 in EGFR-TKI Acquired Resistant Lung Cancer

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Abstract
Background: Targeted drug resistance is a bottleneck in lung cancer treatment. The present study aimed to explore the role of insulin-like growth factor-binding protein 7 (IGFBP7) in epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) acquired resistance.
Method: A retrospective case-control study was conducted in-vitro on cell lines. Cell lines experiments in-vitro and Stable resistant strains of PC9 cells were selected under 5μM gefitinib. IGFBP7 and the related insulin-like growth factor-1 receptor (IGF-1R)/insulin receptor (IR) signaling pathway proteins were detected by western blot. SiRNA interference was used to downregulate IGFBP7 level in the PC9-Gefitinib resistant (PC9-GR) cells. Cell proliferation assay and invasion assay were carried out. The serum level of IGFBP7 and insulin was detected in EGFR-TKI acquired resistant and EGFR-TKI sensitive patients. Quantitative data was expressed as mean ± standard deviation values. Chi-square test was used to compare the baseline clinical characteristics of the patients. One-way analysis of variance was used to detect the differences between groups. A *P*-value<0.05 was considered statistically significant.

Results: The upregulation of IGFBP7, Tyr1135/1136, IR-β, IRS1 and IGF-1R protein was observed. The interference of IGFBP7 slowed down the proliferation rate and weakened the invasion ability of PC9-GR cells. The EGFR-TKI resistant subgroup analysis according to the T790 mutation (T790M) status suggested that IGFBP7's concentration in the T790M negative group was significantly higher than that in the T790M positive group (53.21 ± 49.15 versus 14.37 ± 0.78, $P < 0.05$).
Conclusion: IGFBP7 and insulin-IGF-1R-IR network contributed to EGFR-TKI acquired resistance. IGFBP7 functioned both in T790M positive and T790M negative EGFR-TKI acquired resistance.

Keywords: Insulin-like growth factor binding protein-related protein 1, Targeted drug resistance, Lung neoplasms

Introduction

Approximately 10%–20% non-small-cell lung carcinoma (NSCLC) in western countries and 40%–50% in East Asia harbored activating mutations in epidermal growth factor receptor (EGFR).[1] The discovery of EGFR-tyrosine kinase inhibitor (TKI) was regarded as a landmark finding in the treatment of NSCLC. Epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) first line treatment results in longer progression-free survival, improved health-related quality of life and decreased treatment-related severe side-effects when compared with those who received standard chemotherapy.[2] However, these patients unavoidably develop acquired drug resistance after a median of 10 to 16 months since taking the first or the second generation EGFR-TKI. The resistance mechanisms included the well described T790 mutation (T790M), the activating bypassing pathways, downstream pathway activation and phenotype transformation.[3, 4]

Insulin-like growth factor-1 receptor (IGF-1R) is a key component of signal pathways in cancer. Circulating insulin-like growth factor-1 (IGF-1), insulin-like growth factor-2 (IGF-2) and insulin bind to the IGF-1R, and trigger a signal transduction cascade that leads to proliferation. However, in-vivo, the phase III clinical trial of adding IGF-1R antibody figitumumab to standard chemotherapy failed to increase overall survival in patients with advanced NSCLC.[5] This may probably due to the other adaptive mechanism. The classified insulin-like growth factor-binding proteins (IGFBPs), including IGFBP1-6, exhibiting high affinity for IGF-1. Their canonical function at the cellular level is to impede access of IGF-1 and IGF-2 to their principal receptor IGF-1R, but IGFBPs can also inhibit, or sometimes enhance, IGF-1R signaling.[6] Insulin Growth factor-binding protein 7 (IGFBP7)-15 exhibit a low affinity for IGF-1. These IGFBPs were reclassified as IGFBP-related proteins (IGFBP-rPs), which played important function in cell growth, differentiation and apoptosis. We devoted on the role of IGFBP7 in cancer in the past decades of years[7]. The important function of IGFBP7 in cancer proliferation, differentiation, and apoptosis was uncovered.

The activation of IGF-1R was well defined in the EGFR-TKI resistance cells.[8] Previous studies indicated that acquired resistance to EGFR-TKI in cancer cells is mediated by loss of IGF-binding proteins.[9] In this study, we aimed to explore the role of IGFBP7 in EGFR-TKI acquired resistance.

Materials and Methods

Cell lines, antibodies, and siRNA interference assay

RPMI1640 cell culture medium with 10% fetal bovine serum was used for routine cultivation of EGFR exon 19 deletion (19Del) lung adenocarcinoma cell line PC9 (frozen in our laboratory). Ten nM gefitinib (AstraZeneca, London, UK) was stimulated for 72 hours, gradually increasing the stimulation concentration until 1μM stimulation for 8 months. Stable resistant strains were selected under 5μM gefitinib and stable clones were screened. Stable drug-resistant clones (named PC9-GR) should be cultured in a culture medium without gefitinib for at least 2 weeks before entering the study to rule out drug effects. The antibody of IGFBP7 was purchased from Abcam, MA, USA. The antibody of Phospho-IGF-I Receptor β (Tyr1135/1136), insulin receptor substrate 1 (IRS1), insulin receptor-β (IR-β), IGF-1R, β-actin and horseradish peroxidase-conjugated secondary antibody were purchased from Cell Signal Technology, MA, USA. Using specific IGFBP7 siRNA duplexes (5'-GGUGAAGGUGCCGAGCUAUTT-3', 5'-AUAGCUCGGCACCUUCACCTT-3') (Suzhou GenePharma, Suzhou, China), IGFBP7 was silenced in PC9-GR.

RT‐PCR and Western blot

The primers for IGFBP7 were sense 5'-CACTGGTGCCCAGGTGTACT-3' antisense: 5'-AACCTACGTACCGTGAGTAT-3'; G3PDH sense: 5'-ACCACAGTCCATGCCATCAC-3'; antisense: 5'-TCCACCACCCTGTTGCTGTA-3'. PCR amplification was performed for 5 min at 95˚C, followed by 24 cycles of denaturation (95˚C, 30 s), annealing (59˚C, 30 s) and extension (72˚C, 30 s). Cell lysates were prepared and quantified using a Pierce BCA protein assay kit (Thermo Fisher Scientific, MA, USA). Equal amounts of protein were loaded onto 12% SDS‐polyacrylamide gels, transferred onto nitrocellulose membranes, and subjected to Western blot analysis. Monoclonal anti-β-actin was used as a loading control.

Cell proliferation assay

Cell proliferation was measured using the cell counting kit-8 (Beyotime, Shanghai, China). Cells (3000 cells per well) were seeded in 96-well plates and incubated for 0 h, 24 h,48 h, 72 h, 96 h, 120h. Ten microliters of CCK8 was added to each well at the time of harvest, according to the manufacturer's instructions. Two hours after adding CCK8, cellular viability was determined by measuring the absorbance of the converted dye at 450 nm. Each assay was repeated three times.

Transwell assay

The cells were diluted to 8×10^5/mL with serum-free RPMI-1640 medium, and 100μL cell suspension was added to the upper transwell chamber (Corning, NY, USA), and 600μL medium containing 20% fetal bovine serum was

added to the lower chamber, respectively. The upper chamber was carefully immersed in the lower chamber liquid with sterile forceps. The 24-well plate with transwell chamber was incubated in a 37°C incubator for 24 hours. After 24 hours, the liquid was removed from the upper chamber, and placed in a hole containing 600μL PBS three times. After crystal violet staining, the upper chamber was observed under electron microscope and photographed. Each experiment was repeated three times.

Patients and blood sampling

From January 2017 to September 2018, twenty-nine hospitalized lung cancer patients (17 EGFR-TKI acquired resistant patients, 8 EGFR-TKI sensitive patients, 4 EGFR-wide type patients) were enrolled. Here, EGFR-TKI referred to the first generation EGFR-TKI. Newly diagnosed NSCLC patients with EGFR sensitive mutation who have not received EGFR-TKI treatment were enrolled into the EGFR-TKI sensitive group. Patients who had been taking EGFR-TKI when disease progression was first detected were enrolled into the EGFR-TKI resistance group. Newly diagnosed lung cancer patients without EGFR mutation were enrolled into the EGFR wide type group. Age-matched ten healthy controls were selected from the population on the basis of routine physical examination. Upon agreement of the Medical Ethics Committee of Sir Run Run Shaw Hospital, the study was commenced. Informed consent was obtained from all individual participants included in the study. A venous blood sample was obtained from each patient on the admission day. For biochemical measurements, the supernatants were stored at −70℃ after centrifugation at room temperature at 2,000 × g. The separated serum sample was aliquoted into sterile microcentrifuge tubes (500 μL each vial), then stored at −80℃.

Biochemical measurements

Serum IGFBP7 levels were analyzed using a commercially available ELISA kit (USCN Life Science, Inc., Wuhan, China) according to the manufacturer's protocol. Insulin was detected by chemiluminescence immunoassay (Beckman Coulter DIX800, USA).

Ethics approval

The study was approved by the Medical Ethics Committee of Sir Run Run Shaw Hospital (Date: 2024-Nov-20, Number: 2024-2671-01). Informed consent was waived due to the retrospective design of the study.

Statistical analysis

Quantitative data was expressed as mean ± standard deviation values. Chi-square test was used to compare the baseline clinical characteristics of the patients. One-way analysis of variance was used to detect the differences between groups. A *P*-value < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS for Windows, version 22.0 (IBM Corporation, Armonk, NY, USA)

Results

Establishment of PC9-GR cells

We successfully established a stable gefitinib-resistant NSCLC cell line, named PC9-GR. RT-PCR and western blot showed that the mRNA and the protein expression of IGFBP7 was up-regulated in PC-GR cells (Figure 1A, 1B). The addition of 1μmol/L gefitinib could significantly inhibited the proliferation rate of PC9 cells (*P* < 0.05,

Figure 1C). While the PC9-GR cells showed resistance to 1μmol/L gefitinib (P > 0.05, Figure 1D). The transwell assay showed that the invasion ability of PC9-GR was stronger than PC9 cells (Figure 1E). The relative fold change was 2.05 ± 0.14 (P<0.05, Figure 1F).

The upregulation of IGF-1R- IR signaling pathway in PC9-GR cells

Western blot analysis showed the upregulation of Tyr-1135/1136, IRS1, IR-β, β-actin, IGF-1R protein level was in PC9-GR cells (Figure 2A). The relative fold change was 1.98 ± 0.18, 3.04 ± 0.22, 1.67 ± 0.15, 3.36 ± 0.23, respectively (Figure 2B)

IGFBP7 interference in PC9-GR cells leaded to slower proliferation and weakened invasion

The interference of IGFBP7 was conducted in PC9-GR cells. The interference cell line was named PC9-GR-Si. The protein level of IGFBP7 was significantly decreased in PC9-GR-Si cells (Figure 3A). The relative fold change was 0.38 ± 0.02 (versus PC9-GR-vector, P < 0.05, Figure 3B). The proliferation of PC9-GR-Si cells was slower than PC9-GR cells (Figure 3C, P<0.05).

The transwell assay showed that the invasion ability of PC9-GR-Si cells was weaker (Figure 3D). The relative cell invasion was 0.36 ± 0.02(versus PC9-GR vector, P < 0.05, Figure 3E).

Significant upregulation of IGFBP7 in T790M negative EGFR-TKI acquired resistant patients

The clinicopathologic characteristics of the patients are shown in Table 1. IGFBP7 data from three samples (one EGFR-TKI resistance, one EGFR-sensitive, one EGFR wide type) were missed due to hemolysis. The mean level of IGFBP7 protein was higher in the serum of patients after acquired resistance to the first-generation EGFR-TKI than in that of EGFR-TKI sensitive patients (23.44 ± 28.29ng/ml versus 12.26 ± 2.2ng/ml, Figure 4A), although the statistical significance did not reach, P > 0.05. The mean value of the insulin expression level was higher in the serum of patients after acquired resistance to the first-generation EGFR-TKI than in that of EGFR-TKI sensitive patients (10.25 ± 12.37 μIU/ml versus 4.90 ± 2.50μIU/ml, Figure 4B), although the statistical significance did not reach, P > 0.05. Interestingly, the data in the EGFR-TKI resistance group has a high degree of dispersion. In the EGFR-TKI acquired resistance group, there were two samples with IGFBP7 level >60ng/ml, and three samples with insulin concentration >24μIU/ml. In the control EGFR-TKI sensitive group, the extremely large value did not occur. The IGFBP7 level in the T790M negative EGFR-TKI acquired resistant patients was significantly higher than that in T790M positive patients (53.21 ± 49.15 versus 14.37 ± 0.78ng/ml, Figure 4C, P < 0.05).

Discussion

In the present study, the upregulation of IGFBP7 in PC9-GR cells was observed. The interference of IGFBP7 slowed down the proliferation rate and weakened the invasion ability of PC9-GR cells. The EGFR-TKI resistant subgroup analysis according to the T790 mutation (T790M) status suggested that IGFBP7's concentration in the T790M negative group was significantly higher than that in the T790M positive group.

Different from the previous report for the downregulation of IGFBP3 and IGFBP4,[9] we demonstrated the upregulation of IGFBP7 in gefitinib resistant lung cancer PC9-GR cell lines. The transient addition of gefitinib did not induce increase the expression of IGFBP7（data not shown）, suggesting that the upregulated level of IGFBP7 in PC9-GR cells was correlated with drug resistance. After interference of IGFBP7 in PC9-GR cells, the proliferation rate slowed down and invasion ability weakened, indicating that IGFBP7 plays an important role in EGFR-TKI acquired resistance. Our findings are in consistent with Wu and Tang et al.[10,11, 13] Wu et al. collected four gene expression datasets consisting of 15 experiments to compare EGFR-TKI-sensitive to EGFR-TKI-resistant cells, and calculated the TKI resistance-related score for each gene based on its expression. As a result, IGFBP7 was ranked as the top TKI resistance-related gene.[10] This interesting finding was verified in our study.

PC9-GR cells were reported to harbor mutation of T790.[12] Here, we demonstrated the acquired gefitinib resistance related role of IGFBP7. Whether this phenomenon still existed if T790M was conquered by the 3rd generation EGFR-TKI is of great concern. Osimertinib is currently indicated as second-line therapy in NSCLC patients who present T790M after treatment with previous 1st or 2nd EGFR-TKIs or the first-line therapy in patients with sensitizing EGFR mutations.[13] Interestingly, Tang et al. reported that IGFBP7 overexpression promoted acquired resistance to osimertinib in NSCLC. These researches strongly indicated the exact role of IGFBP7 in EGFR-TKI resistance. We detected elevated level of IGFBP7 in the serum of EGFR-TKI resistant patients. The significant difference did not reach perhaps due to the small samples. Large patient samples should be collected in the future. The very large dispersion of the data attracted our attention. The subgroup according to the T790M status suggested that IGFBP7 concentration in the T790 negative group was significantly higher than that in the T790M positive group. Our data indicated the IGFBP7's probable important role in the T790M negative EGFR-TKI acquired resistance, which should further be investigated.

Different from IGFBP1-6, IGFBP7 has low binding affinity to IGF-1R and high binding affinity to IR.[14] The protein cooperates in regulating signals from IRs and IGF receptors.[7] We demonstrated the upregulation of IGF-1R, the Phospho-IGF-1 Receptor β（Tyr1135/1136）, and the downstream IRS1, indicating the activation of IGF-1R pathway in EGFR-TKI resistance. The role of IR in EGFR-TKI resistant lung cancer was not well defined in previous studies. We first reported the upregulation of IR-β in PC9-GR cells, indicating the probable important role of IGFBP7-IR-IGF-1R network in EGFR-TKI resistance. Besides lung cancer, the interesting phenomenon was also found in other cancer cells. Guix et al. reported that to escape EGFR-TKI treatment, cholangiocarcinoma cells develop an adaptive mechanism by undergoing an IR/IGF-1R-dependent phenotypic switch,

involving a contribution of stromal cells.[9]

We detected the elevated level of insulin in the serum of EGFR-TKI resistant patients. The significant difference did not reach perhaps due to the small samples. Large patient samples should be collected in the future. When we searched PUBMED, only one paper had reported the relation between hyperinsulinemia and EGFR-TKI resistance. Chiu et al. found hyperinsulinemia but not hyperglycemia, was identified to cause the development of gefitinib resistance in NSCLC cells with activating EGFR mutations.[15] Metformin is a widely used drug against type 2 diabetes, which could improve insulin's action on whole-body glucose metabolism in various insulin-resistant populations.[16] Experiments from different laboratories reported that metformin sensitized EGFR-TKI-resistant human lung cancer cells.[17-18] Beloueche-Babari et al. reported that acquired resistance to EGFR-TKI alters the metabolism of human head and neck squamous carcinoma cells and xenograft tumors. These evidences together with ours indicated that to conquer EGFR-TKI resistance, not only the cell signaling pathway, but also the metabolic environment should be considered. To conquer cancer, the cancer foci and the surrounding microenvironment should both be targeted. Our study suggests the important role of IGFBP7-insulin-IGF-1R-IR network in EGFR-TKI resistant field. However, our study has certain limitations. First, we used only one lung cancer cell lines. The exact molecular mechanism after IGFBP7 knockdown should be further conducted in the future. Second, the clinical samples were small. Third, we used only first-generation EGFR-TKI. The widely used third-generation-EGFR-TKI was not researched in our study. Further investigation with large samples may provide valuable evidence.

Conclusion

In this study, we found the upregulation of IGF-1R- IR signaling pathway in PC9-GR cells. The interference of IGFBP7 slowed down the proliferation rate and weakened the invasion ability of PC9-GR cells. IGFBP7's concentration in the T790M negative group was significantly higher than that in the T790M positive group. Our data suggests the important role of IGFBP7 and insulin-IGF-1R-IR network in EGFR-TKI acquired resistance. The exact role of IGFBP7 in T790M positive and T790M negative EGFR-TKI acquired resistance will further be explored.

Data Availability

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

Authors' Contribution

HQG contributed in data analysis, writing original draft; RWJ contributed in study design, data collection and analysis, serum collection, revising original draft; ZHQ contributed in cell culture, siRNA assay, western blot and ; YC contributed in the serum collection and ELISA; XYM contributed in RT-PCR and western blot. All authors reviewed it critically for important

intellectual content, finally approved the version to be published. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Conflict of Interest
None declared.

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Table 1. Clinicopathologic Characteristics of the EGFR-TKI acquired and sensitive patients

	EGFR-TKI acquired resistant	**EGFR-TKI sensitive**	**EGFR wide type**	**Normal control**	***P* value (resistant versus sensitive)**
Cases	17	8	4	10	N/A
Age, median (IQR)	61(55-72)	72.5(66.75-79)	71(69.75-76.5)	68(64.5-69.75)	*P* = 0.05
Sex					*P* = 0.68
Female	10(58.8%)	4(50%)	0(0%)	3(30%)	
Male	7(41.2%)	4(50%)	4(100%)	7(70%)	
Hypertension	6(35.3%)	1(12.5%))	3(75%)	2(20%)	*P* = 0.24
Diabetes	2(11.8%)	0(0%)	0(0%)	0(0%)	*P* = 0.34
IGFBP7(ng/ml)	23.44 ± 28.29	12.26 ± 2.20	14.0 ± 2.79	16.80 ± 18.85	*P* = 0.31
Insulin(μIU/ml)	10.25 ± 12.37	4.90 ± 2.50	8.60 ± 7.06	3.21 ± 1.37	*P* = 0.24
T790M positive	8	N/A	N/A	N/A	N/A
T790M negative	4	N/A	N/A	N/A	N /A
T790M unknown	5	N/A	N/A	N/A	N/A

EGFR: Epidermal growth factor receptor; TKI: Tyrosine Kinase Inhibitor; IQR: Interquartile range

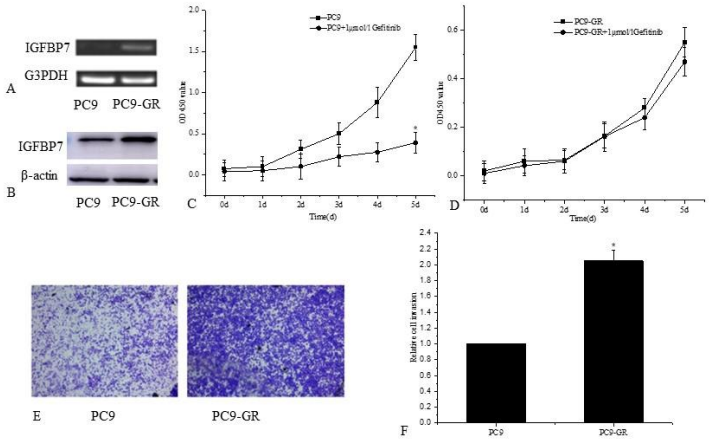

Figure 1. This figure shows the upregulation of IGFBP7 in PC9-GR cells. A: RT-PCR showed the upregulation of IGFBP7 mRNA in PC9-GR cells. B. western blot showed the upregulation of IGFBP7 protein in PC9-GR cells. C. The growth curve of PC9 cells with or without 1μmol/L gefitinib, $^{*}P < 0.05$. D. The growth curve of PC9-GR cells with or without 1μmol/L gefitinib, $P > 0.05$. E. Cell invasion was assessed by transwell assay. Representative photographs were presented (×400 magnification, crystal violet staining). F. The relative cell invasion of PC9-GR cells (relative to PC9 cells) $^{*}P < 0.05$.

IGFBP7: Insulin-like growth factor binding protein 7; GR: Gefitinib resistant; RT-PCR: Reverse transcription polymerase chain reaction

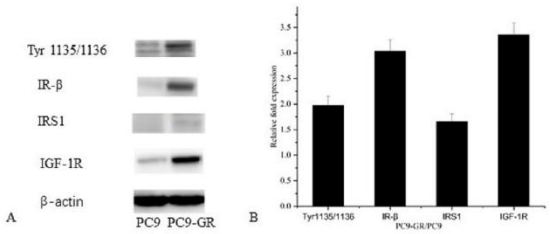

Figure 2. This figure shows the expression of IGF-1R/IR signaling proteins in PC9-GR cells. A. Western blot showed the upregulation of Tyr-1135/1136, IR-β, IRS1, IGF-1R in PC9-GR cells. B: The relative fold change of the protein expression of Tyr-1135/1136, IR-β, IRS1, IGF-1R in PC9-GR cells relative to PC9 cells.

IGF-1R: Insulin-like growth factor-1 receptor; IR: Insulin receptor; GR: Gefitinib resistant; IRS-1: Insulin receptor substrate-1

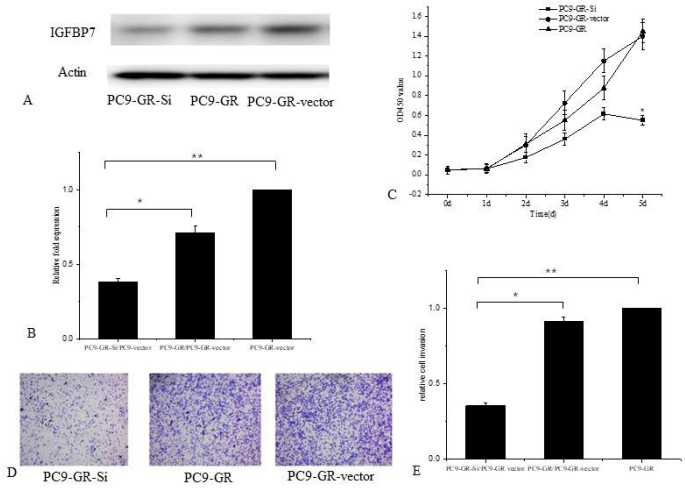

Figure 3. This figure shows the downregulation of IGFBP7 inhibited cell proliferation and weakened invasion ability of PC9-GR cells. A SiRNA interference downregulated the IGFBP7 protein level in PC9-GR cells. B. The relative fold change of IGFBP7 protein level in PC9-GR-Si, PC9-GR, PC9-GR-vector cells, *,** $P < 0.05$. C. The growth curve of PC9-GR-Si, PC9-GR, PC9-GR-vector cells. $^{*}P < 0.05$, PC9-GR-Si versus PC9-GR vector cells D. Cell invasion was assessed by transwell assay. Representative photographs were presented (×400 magnification, crystal violet staining) E. The relative cell invasion of PC9-GR-Si cells. *,** $P < 0.05$.

IGFBP7: Insulin-like growth factor binding protein 7; GR: Gefitinib resistant; Si: Small interfering

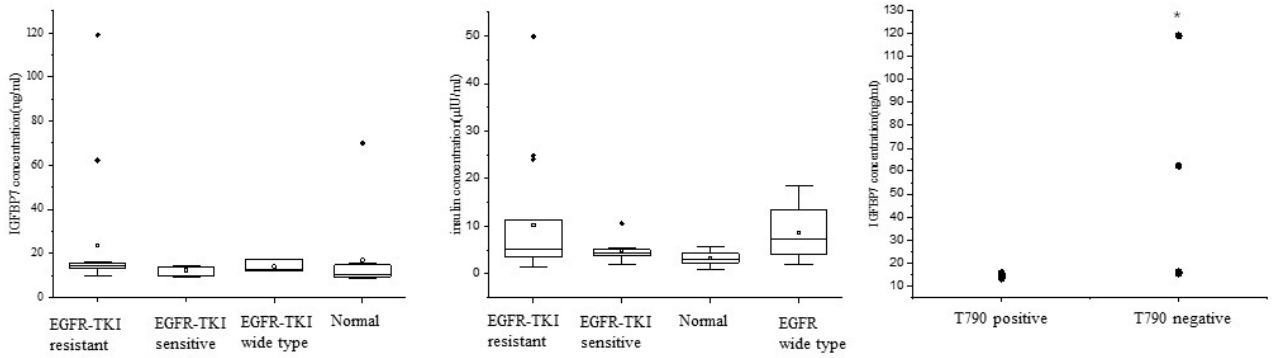

Figure 4. This figure shows the expression change of IGFBP7 and insulin in the EGFR-TKI acquired resistant patients. A. The mean IGFBP7 level was higher in the serum of patients after acquired resistance to EGFR-TKI than that of EGFR-TKI sensitive patients, although the statistical significance did not reach, $P > 0.05$. B. The mean insulin level was higher in the serum of patients after acquired resistance to EGFR-TKI than in that of EGFR-TKI sensitive patients, although the statistical significance did not reach, $P > 0.05$. C. IGFBP7 concentration in the T790M negative EGFR-TKI acquired resistance group is significantly higher than that in the T790M positive EGFR-TKI acquired resistance group, $^{*}P < 0.05$.

▫ mean data; IGFBP7: Insulin-like growth factor binding protein 7; EGFR: Epidermal growth factor receptor; TKI: Tyrosine kinase inhibitor; T790M: T790 mutation