Small molecule TKI inhibitors affect the development of non-small cell carcinoma through HIPPO/YAP/PD-L1

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ABSTRACT
Non-small cell lung cancer accounts for approximately 80%–85% of lung cancer (1, 2). With the rapid development of medical technology, targeted therapy is a common choice in treatment plans due to its low toxicity and high pertinence (3). EGFR can reduce the possibility of autophagy by activating downstream factors such as P13K and MAPK (4, 5). TKI inhibitors are targeted therapies for EGFR receptors, which bind to receptor target genes to block downstream pathways and improve tumor development (6, 7). Low expression of membrane proteins on PD-1T cells is an important molecule in regulating the autoimmune system (8, 9). Existing literature shows that the expression of PD-L1 is increased in tumor patients, and this protein can combine with PD-1, resulting in the occurrence of immune escape (10). The activation of YAP can be involved in tumor development. Studies have confirmed that the increased expression of YAP in serum and tissues of lung cancer patients can promote cell proliferation and metastasis (11, 12), so most drugs can inhibit tumor proliferation through Hipo-YAP. At present, there are few studies on TKI inhibition and its relationship with Hipo-YAP or PD-L1 at home and abroad. Therefore, in order to explore the correlation among the three and provide a reference for clinical drug use, this study took non-small cell lung cancer A549 cell line as the research object to explore the relationship between the influence of small molecule inhibitors on the development of non-small cell carcinoma and HIPPO/YAP/PD-L1 signaling pathway, so as to provide a basis for tumor targeting and immunotherapy. The results are as follows.

Introduction
Non-small cell lung cancer accounts for approximately 80%–85% of lung cancer (1, 2). With the rapid development of medical technology, targeted therapy is a common choice in treatment plans due to its low toxicity and high pertinence (3). EGFR can reduce the possibility of autophagy by activating downstream factors such as P13K and MAPK (4, 5). TKI inhibitors are targeted therapies for EGFR receptors, which bind to receptor target genes to block downstream pathways and improve tumor development (6, 7). Low expression of membrane proteins on PD-1T cells is an important molecule in regulating the autoimmune system (8, 9). Existing literature shows that the expression of PD-L1 is increased in tumor patients, and this protein can combine with PD-1, resulting in the occurrence of immune escape (10). The activation of YAP can be involved in tumor development. Studies have confirmed that the increased expression of YAP in serum and tissues of lung cancer patients can promote cell proliferation and metastasis (11, 12), so most drugs can inhibit tumor proliferation through Hipo-YAP. At present, there are few studies on TKI inhibition and its relationship with Hipo-YAP or PD-L1 at home and abroad. Therefore, in order to explore the correlation among the three and provide a reference for clinical drug use, this study took non-small cell lung cancer A549 cell line as the research object to explore the relationship between the influence of small molecule inhibitors on the development of non-small cell carcinoma and HIPPO/YAP/PD-L1 signaling pathway, so as to provide a basis for tumor targeting and immunotherapy. The results are as follows.
Materials and methods

Research Materials
The non-cell lung cancer cell line A549 was obtained from Yuanyuan Biological Co., LTD. TKI inhibitor gefitinib; DMEM medium, Trizol lysate, real-time quantitative PCR reagent. Cell transfection reagents were all from Hangzhou Zhonuo Biotechnology Co., LTD.

Routine A549 tumor cell culture
Non-small cell carcinoma A549 cell line was selected and divided into 4 groups, namely group A (TKI 100nmol/l), group B (TKI 200nmol/l), group C (TKI 300 nmol/l) and normal saline group.
Cell transfection groups were transfected into A549 cells with YAP shRNA and established into the YAP shRNA group and NC group. Both groups were treated with TKI 200nmol/L.

Cell proliferation was determined by the CCK-8 method
The ckk-8 assay was used to detect the proliferation of A549 and its transfected cells under the intervention of different concentrations of TKI inhibitors. A549 single-cell suspension was prepared. A 96-well plate was used to adjust the number of cells in the suspension to 5×10³ and inoculate each well, and 100, 200 and 300nmol/l TKI inhibitors were added to each well. After 2d, each well was incubated with +10ul CCK-8 for 4h, and the absorbance value at 450nm was measured with a microplate reader.

Apoptosis was determined by Annexin V-FITC/PI double staining
The cells in the above groups were prepared into 1×10⁶ cells/ml cell suspension, and then 400ul binding buffer +5ul PI+Annexin v-fitic solution was added. After 15min of incubation, apoptosis was determined by up-flow cytometry.

Dual-luciferase reporting system
Targetscan biological software was used to predict the related targets of YAP, and it was found that there was a narrow peak TEAD4 binding site 13kb upstream of the pdL-1 transcription start site. Therefore, the luciferase reporter system was used to determine the targeting relationship between the two, and luciferase reporter vectors containing YAP-TeAD4 and luciferase reporter vectors containing mutated YAP-TeAD4 were established and labeled WT and MUT, respectively. Then, YAP blank and YAP mimics were transfected into cells, respectively, and the luciferase concentration was determined with the corresponding kit.

RT-PCR
Conventional total RNA extraction, cDNA reverse transcription and PCR reaction were performed. GAPDH was used as the internal reference protein, and relative quantitative analysis was performed with 2ΔΔCT. Specific amplitizer parameters were carried out according to the corresponding instructions, and primer sequences were shown in Table 1.

Table 1. Primer sequences used in this expriment

| Primer | Upstream primer | Downstream primers |
|--------|----------------|--------------------|
| YAP    | GCTTGTTCATCC   | GCAGGTTTGGGAGAT    |
| PD-L1(14) | ATCAGGAGAG | GGCACAGGAC         |
| Vimentin(15) | CTGCATGATCAGC | GATTACCGGCACG     |
| Bcl-2(16) | GGATGCTCGCTG | GCTGCCGA          |
| GAPDH  | GAGTCAACGGATT | TGGTTCGT          |

Western BLOT detection
A549 cells were collected routinely, cell lysates were collected, and total protein content was determined using BCA protein quantitative kit. 50ug total protein was placed on SDS-Page, and the classified protein was transferred to PVDF membrane by the wet membrane transfer device. The membrane was sealed with 5% skim milk powder solution at 4°C overnight, and the primary anti-YAP and PD-L1 antibodies were added after the membrane was washed with TNST buffer solution. After being sealed at room temperature for 2h, TBST solution was cleaned, secondary antibody was added, and incubated for 1h without light before TBST solution was cleaned. After that, the absorbance value of the bands was analyzed by gel imaging system.

Statistical analysis
SPSS20.0 was used for statistical processing of the data in this study, and all data were expressed as standard deviation ± mean. T-test was used for.
comparison of mean between two groups, and F test was used for comparison between multiple groups. P<0.05 was considered as a statistically significant difference.

Results and discussion
Effects of TKI inhibitors on survival rate and apoptosis rate of A549 cells
In terms of cell apoptosis rate, group A < group B < group C, and in terms of cell survival rate, group A > group B > group C, the differences among the three groups were statistically significant (P<0.05), as shown in Figure 1.

![Figure 1. Effects of TKI inhibitors on proliferation and apoptosis of A549 cells. Note: Compared with Group A, ***P<0.001; compared with Group B, ##P<0.01](image)

Effects of TKI inhibitors on the expression of A549 cell-related proteins
In terms of protein expression of YAP, PD-L1, Bcl-2 and Vimentin, group A > group B > group C showed statistical significance (P<0.05), as shown in Table 2.

Table 2. Effects of TKI inhibitors on the expression of A549 cell-related proteins

| Group | Group A | Group B | Group C | F value | P value |
|-------|---------|---------|---------|---------|---------|
| YAP   | 4.63±0.21 | 3.54±0.48 | 2.64±0.37 | 4.312   | 0.035   |
| PD-L1 | 1.92±0.13  | 1.46±0.19  | 1.12±0.14  | 5.149   | 0.011   |
| BCL-2 | 1.46±0.45  | 0.93±0.41  | 0.68±0.21  | 6.485   | 0.014   |
| Vimentin | 2.68±0.52 | 2.05±0.46  | 1.76±0.11  | 5.425   | 0.018   |

Effects of YAP overexpression on proliferation and apoptosis of A549 cells
The apoptosis rate of the shRNA group was lower than that of the NC group, and the cell survival rate was significantly higher than that of the NC group, with statistical significance (P<0.05), as shown in Figure 2.

![Figure 2. Effects of YAP overexpression on proliferation and apoptosis of A549 cells. Note: Compared with Group NC, **P<0.01](image)

Effects of YAP overexpression on the expression of A549 cell-related proteins
The expression of YAP, PD-L1, Bcl-2 and Vimentin in the shRNA group was significantly higher than that in the NC group, with statistical significance (P<0.05), as shown in Table 3.
Table 3. Effects of YAP overexpression on the expression of A549 cell-related proteins

| Group     | Group shRNA | Group NC | T-value | P-value |
|-----------|-------------|----------|---------|---------|
| YAP       | 4.59±0.45   | 3.62±0.39| 6.714   | 0.016   |
| PD-L1     | 1.84±0.18   | 1.50±0.09| 7.419   | 0.011   |
| BCL-2     | 1.65±0.23   | 1.01±0.22| 8.416   | 0.007   |
| Vimentin  | 2.89±0.45   | 2.13±0.28| 7.419   | 0.011   |

Luciferase determination

On MUT, there was no statistically significant difference between the NC group and the YAP group (P>0.05); on WT, compared with the NC group, the luciferase activity in the YAP group was significantly decreased (P<0.05), as shown in Figure 3.

Lung cancer originates from bronchial mucosal epithelium, and the vast majority of patients are non-small cell carcinoma, while the remaining patients are small cell lung cancer (17). Up to now, more than 50 TK receptors have been discovered, among which EGFR TK receptors include HER1~4. HER1 can bind to growth factors and lead to TK phosphorylation, which in turn activates MAPK, STAT3 and other signaling factors (18). The main intervention mechanism of small molecule TKI inhibitors involved in tumor development (19-21) is to block EGFR expression by binding to the extracellular EGFR target of T cells, thereby weakening tumor development and achieving the therapeutic goal (22, 23).

BCL2 is closely related to tumor cell survival, and the increase of its protein level may indicate tumor progression (24). Vimentin protein is a common marker in the EMT process of tumor cells (25). The results of this study indicated that TKI inhibitors could inhibit the proliferation and migration of A549 cells, promote apoptosis, and inhibit HIPPO-related pathways, which was positively correlated with concentration (26). Zhao et al. (27) in a meta-analysis, oxitinib and gefitinib plus pemetrexed-based chemotherapy were associated with optimal progression-free survival and overall survival benefits in patients with advanced EGFR-mutated NSCLC compared with other first-line therapies. Xu et al. (28) showed that upregulation of Hippo-TAz can lead to resistance of lung cancer cells carrying EGFR-T790m mutation to gefitinib. Dent et al. (29) showed that lenatinib synergistically inhibited the functions of mutated K-RAS and YAP to kill pancreatic tumor cells. The above results are consistent with the results of this study, which all confirmed the obvious inhibitory effect of TKI inhibitors on NON-small cell lung cancer, and the main mechanism is related to the HIPPO signaling pathway. Among them, the mechanism of TKI inhibitor inhibiting tumor cell proliferation has been described, while HIPPO signaling pathway consists of MST1/2, LATS1/2, YAP/YAZ, etc. It has been confirmed that YAP/YAZ is a pathogenic gene of lung cancer, which is located on human chromosome 11Q22 and overexpressed in tumors (30). YAP is an isoform of YAZ, and the increase of YAP is closely related to the enhancement of tumor cell proliferation and can induce the EMT process in lung cancer, thus leading to the increase of BCL2 and Vimentin in this study (31, 32).

The results of this study showed that compared with NC group, the cell survival rate of the shRNA group was significantly increased and the cell apoptosis rate was significantly decreased. The expression of YAP, PD-L1, Bcl-2 and Vimentin in the shRNA group was significantly higher than that in the NC group. Dual-luciferase gene analysis results indicated that YAP and PD-L1 were in a targeted regulatory relationship, specifically, the combination of YAP and PD-L1 promoted the expression of PD-L1. Compared with the NC group, there was no significant difference between MUT in the YAP group, WT luciferase activity in the YAP group decreased significantly, suggesting that YAP overexpression can reverse the inhibitory effect of TKI inhibitor on A549 cell proliferation and promote apoptosis. Moreover, YAP is an upstream target factor of PD-L1, and the combination of the two can promote the expression of PD-L1. Miao et al. (33) found that in (1) NON-small...
In conclusion, small-molecule TKI inhibitors can significantly inhibit proliferation and migration of non-small cell carcinoma and promote apoptosis, and the specific mechanism is related to the inhibition of the HIPPO/YAP/PD-L1 signaling pathway.

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None.

Interest conflict
The authors declare no conflict of interest.

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