



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

Li Lei, Zhang Weidong*

Department of Thoracic Surgery, Tianjin First Central Hospital, Tianjin 300192, China

ARTICLE INFO

Original paper

Article history:

Received: August 17, 2021

Accepted: November 16, 2021

Published: December 15, 2021

Keywords:

Tyrosine kinase inhibitor;
HIPPO signaling pathway;
Non-small cell carcinoma;
Programmed cell death protein-1 ligand

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.

DOI: <http://dx.doi.org/10.14715/cmb/2021.67.5.16>

Copyright: © 2021 by the C.M.B. Association. All rights reserved.



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.

*Corresponding authors: Weidongz@foxmail.com

Cellular and Molecular Biology, 2021, 67(5): 117-122

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 cck-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 5×10^3 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 1×10^6 cells /ml cell suspension, and then 400ul binding buffer +5ul PI+Annexin v-fitc 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 $2^{-\Delta\Delta CT}$. Specific amplifizer parameters were carried out according to the corresponding instructions, and primer sequences were shown in Table 1.

Table 1. Primer sequences used in this experiment

Primer	Upstream primer	Downstream primers
YAP ⁽¹³⁾	GCTTGTCCCATCC ATCAGGAAG	GCAGTTGGGAGAT GGCAAAGAC
PD-L1 ⁽¹⁴⁾	CTGCATGATCAGC TATGGTGGTG	CAGTTCATGTTTCAGA GGTGACTGGA
Vimentin ⁽¹⁵⁾	AGATATCTCGCTG CGTCGAA	GATTACGCGTCACG CGTCCGAA
Bcl-2 ⁽¹⁶⁾	GGGATGCCTTTGT GGAAC	GTCTGCTGACCTCAC TTG
GAPDH	GAGTCAACGGATT TGGTCGT	TTGATTTGGAGGGA TCTCG

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 \pm 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.

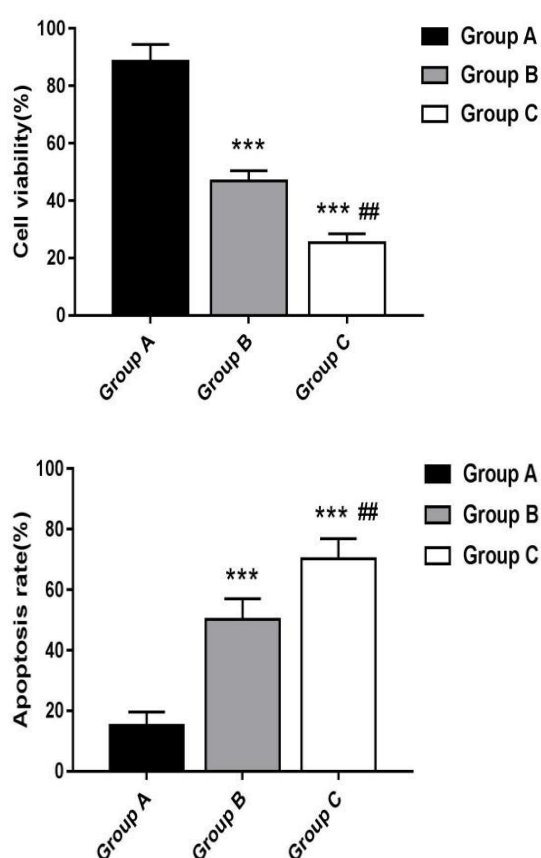


Figure 1. Effects of TKI inhibitors on proliferation and apoptosis of A549 cells. Note: Compared with Group A, *** $P < 0.001$; compared with Group B, ## $P < 0.01$

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.

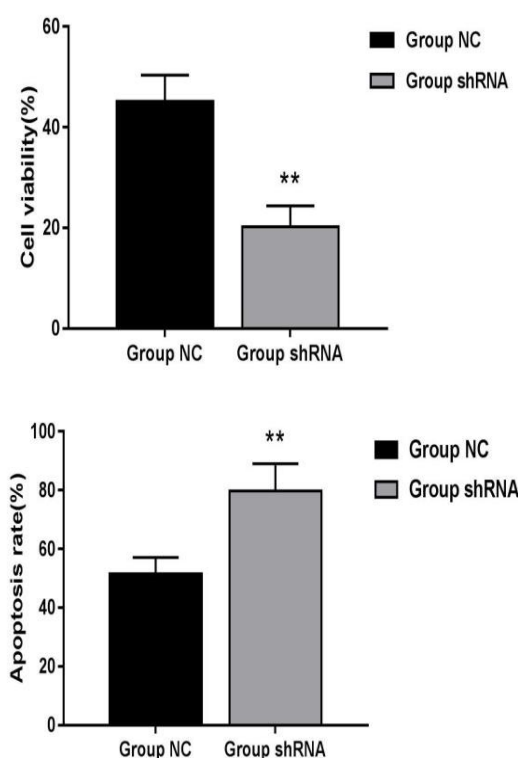


Figure 2. Effects of YAP overexpression on proliferation and apoptosis of A549 cells. Note: Compared with Group NC, ** $P < 0.01$

Effects of YAP overexpression on the expression of A549 cell-related proteins

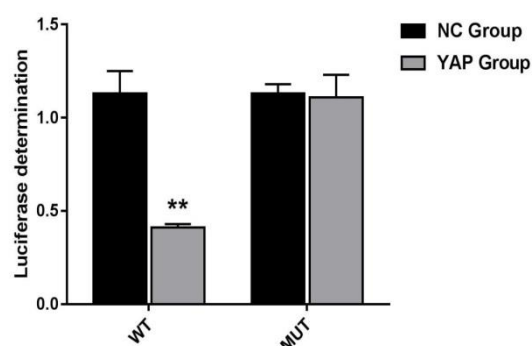
The expression of YAP, PD-L1, Bcl-2 and Vimentin in the shRNA group was significantly higher than that in the NC group, with statistical significance ($P < 0.05$), as shown in Table 3.

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.

**Figure 3.** Dual-luciferase reporting experiment. Note: Compared with Group NC, ** $P<0.01$

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

cell lung cancer tissues, immunohistochemistry showed positive staining of YAP and PD-L1, which were significantly correlated. (2) The ratio of P-YAP to YAP was lower in cell lines with high pD-L1 expression (H460, SKLU-1 and H1299), and the GTIIC reporter gene activity of Hippo pathway was higher than that of pD-L1 with low PD-L1 expression (A549, H2030 and PC9). (3) Forced overexpression of YAP saved PD-L1 mRNA and protein levels after siRNA knockdown of 3'UTR targeting endogenous YAP gene. Lee et al. (34) showed that YAP knockdown significantly reduced the expression of PD-L1 in EGFR-TKI resistant cells, while YAP overexpression increased the expression of PD-L1 in parental PC9 cells. YAP regulates the transcription of PD-L1, and the YAP/TEAD complex binds to the PD-L1 promoter. In EGFR-TKI-resistant PC9 cells, the down-regulation of PD-L1 was sufficient to reduce cell proliferation and wound healing, suggesting that PD-L1 has a carcinogenic function independent of PD1. While Hippo effector YAP plays a key role in connecting PD-L1 and EGFR-TKI resistance by directly regulating the expression of PD-L1 in lung cancer. The above results are consistent with the results of this study, which all confirm that YAP can regulate the transcription of PD-L1. TKI inhibitors can reduce the expression of PD-L1 by inhibiting the Hippo-YAP signaling axis, thus regulating the development of NON-small cell lung cancer. 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.

Acknowledgements

None.

Interest conflict

The authors declare no conflict of interest.

References

1. Villalobos P, Wistuba II. Lung cancer biomarkers. *Hematol Oncol Clin* 2017; 31(1): 13-29.
2. Sutanto YS, Ibrahim D, Septiawan D, Sudiyanto A, Kurniawan H. Effect of Cognitive Behavioral Therapy on Improving Anxiety, Depression, and Quality of

Life in Pre-Diagnosed Lung Cancer Patients. *Asian Pac J Cancer Prev* 2021; 22(11): 3455-3460.

3. Ruiz-Cordero R, Devine WP. Targeted therapy and checkpoint immunotherapy in lung cancer. *Surg Pathol Clin* 2020; 13(1): 17-33.

4. Yoneda K, Imanishi N, Ichiki Y, Tanaka F. Treatment of non-small cell lung cancer with EGFR-mutations. *J UOEH* 2019; 41(2): 153-163.

5. Mehdi SH, Zafaryab M, Nafees S et al. Chrysin sensitizes human lung cancer cells to tumour necrosis factor related apoptosis-inducing ligand (TRAIL) mediated apoptosis. *Asian Pac j cancer biol* 2019; 4(2): 27-33.

6. Huang L, Jiang S, Shi Y. Tyrosine kinase inhibitors for solid tumors in the past 20 years (2001–2020). *J Hematol Oncol* 2020; 13(1): 1-23.

7. Jung M. A Legal and Epidemiological Consideration of the Causal Relationship between Tobacco and Lung Cancer. *Asian Pac J Cancer Prev* 2021; 22(9): 2723-2727.

8. Xia L, Liu Y, Wang Y. PD-1/PD-L1 blockade therapy in advanced non-small-cell lung cancer: current status and future directions. *Oncol* 2019; 24(Suppl 1): S31.

9. Ercisli MF, Kahrizi D, Aziziaram Z. Environmental factors affecting the risk of breast cancer and the modulating role of vitamin D on this malignancy. 2020.

10. Yang Y-L, Li Z-Q, Wang Q-L, Gu J-J, Fang X-J, Huang G-H. Efficacy and Safety of Programmed Cell Death 1 Inhibitor Monotherapy Versus Chemotherapy as Second-Line Treatment for Advanced Esophageal Cancer: A Meta-analysis and Systematic Review. *Clin Therapeut* 2021; 43(11): 1997-2012.

11. Lo Sardo F, Strano S, Blandino G. YAP and TAZ in lung cancer: oncogenic role and clinical targeting. *Cancers* 2018; 10(5): 137.

12. Musika W, Kamsa-Ard S, Jirapornkul C, Santong C, Phunmanee A. Lung Cancer Survival with Current Therapies and New Targeted Treatments: A Comprehensive Update from the Srinagarind Hospital-Based Cancer Registry from (2013 to 2017). *Asian Pac J Cancer Prev* 2021; 22(8): 2501.

13. Zhou G, Xie J, Gao Z, Yao W. MicroRNA-877 inhibits cell proliferation and invasion in non-small cell lung cancer by directly targeting IGF-1R. *Exp Ther Med* 2019; 18(2): 1449-1457.

14. Carvajal-Hausdorf D, Altan M, Velcheti V et al. Expression and clinical significance of PD-L1, B7-H3, B7-H4 and TILs in human small cell lung cancer (SCLC). *J Immunother Cancer* 2019; 7(1): 1-9.
15. Kim TW, Lee YS, Yun NH et al. MicroRNA-17-5p regulates EMT by targeting vimentin in colorectal cancer. *Br J Cancer* 2020; 123(7): 1123-1130.
16. Ran Z, Feng Q, Song Y et al. Expression and relationship of Beclin1 and Bcl2 in invasive pituitary adenomas. *Chin J Endocrine Surg* 2012; 6(4): 253-256.
17. Torre LA, Siegel RL, Jemal A. Lung cancer statistics. *Lung Cancer Person Med* 2016: 1-19.
18. Choudhari C, Bharati SJ, Hoda W, Kumar S, Kumar S, Shankar A. Dilemma and Strategies Amidst Nationwide Covid-19 Lockdown for Lung Cancer Management. *Asian Pac j cance care* 2020; 5(S1): 109-112.
19. Harrison PT, Vyse S, Huang PH. Rare epidermal growth factor receptor (EGFR) mutations in non-small cell lung cancer. Paper presented at: Seminars in cancer biology, 2020.
20. Lyu H, Han A, Polsdofer E, Liu S, Liu B. Understanding the biology of HER3 receptor as a therapeutic target in human cancer. *Acta Pharm Sinica B* 2018; 8(4): 503-510.
21. Wang Y, Jiang T, Qin Z et al. HER2 exon 20 insertions in non-small-cell lung cancer are sensitive to the irreversible pan-HER receptor tyrosine kinase inhibitor pyrotinib. *Annal Oncol* 2019; 30(3): 447-455.
22. Sabbah DA, Hajjo R, Sweidan K. Review on epidermal growth factor receptor (EGFR) structure, signaling pathways, interactions, and recent updates of EGFR inhibitors. *Curr Top Med Chem* 2020.
23. Ercisli MF, Lechun G, Azeez SH, Hamasalih RM, Song S, Azizaram Z. Relevance of genetic polymorphisms of the human cytochrome P450 3A4 in rivaroxaban-treated patients. *Cell Mol Biomed Rep* 2021; 1(1): 33-41.
24. Roberts AW. Therapeutic development and current uses of BCL-2 inhibition. *Hematology Am Soc Hematol Educ Program* 2020; 2020(1): 1-9.
25. Tian H, Lian R, Li Y et al. AKT-induced lncRNA VAL promotes EMT-independent metastasis through diminishing Trim16-dependent Vimentin degradation. *Nat Commun* 2020; 11(1): 1-15.
26. Bilal I, Xie S, Elburki MS, Azizaram Z, Ahmed SM, Jalal Balaky ST. Cytotoxic effect of diferuloylmethane, a derivative of turmeric on different human glioblastoma cell lines. *Cell Mol Biomed Rep* 2021; 1(1): 14-22.
27. Zhao Y, Liu J, Cai X et al. Efficacy and safety of first line treatments for patients with advanced epidermal growth factor receptor mutated, non-small cell lung cancer: systematic review and network meta-analysis. *Bmj* 2019; 367.
28. Xu W, Wei Y, Wu S et al. Up-regulation of the Hippo pathway effector TAZ renders lung adenocarcinoma cells harboring EGFR-T790M mutation resistant to gefitinib. *Cell Biosci* 2015; 5(1): 1-9.
29. Dent P, Booth L, Roberts JL et al. Neratinib inhibits Hippo/YAP signaling, reduces mutant K-RAS expression, and kills pancreatic and blood cancer cells. *Oncogene* 2019; 38(30): 5890-5904.
30. Sardo FL, Pulito C, Sacconi A et al. YAP/TAZ and EZH2 synergize to impair tumor suppressor activity of TGFBR2 in non-small cell lung cancer. *Cancer lett* 2021; 500: 51-63.
31. Song J, Xie LX, Zhang XY et al. Role of YAP in lung cancer resistance to cisplatin. *Oncol Lett* 2018; 16(3): 3949-3954.
32. Hsu P-C, Jablons DM, Yang C-T, You L. Epidermal growth factor receptor (EGFR) pathway, yes-associated protein (YAP) and the regulation of programmed death-ligand 1 (PD-L1) in non-small cell lung cancer (NSCLC). *Int J Mol Sci* 2019; 20(15): 3821.
33. Miao J, Hsu P-C, Yang Y-L et al. YAP regulates PD-L1 expression in human NSCLC cells. *Oncotarget* 2017; 8(70): 114576.
34. Lee BS, Park DI, Lee DH et al. Hippo effector YAP directly regulates the expression of PD-L1 transcripts in EGFR-TKI-resistant lung adenocarcinoma. *Biochem Biophys Res Commun* 2017; 491(2): 493-499.