Interleukin 7 Inhibit Autophagy via P53 Regulated AMPK/mTOR Signaling Pathway in Non-small Cell Lung Cancer

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Abstract

Interleukin 7(IL-7) has been demonstrated regulating lymphangiogenesis, apoptosis, and proliferation. Whether IL-7 induce or inhibit autophagy in non-small cell lung cancer(NSCLC) are unknown. Methods In this study, Western blot was used to detect cytoplasmic and nuclear protein of p53, total protein of AMP-activated protein kinase(AMPK) and mammalian target of rapamycin (mTOR). Quantitative Real-Time PCR(qRT-PCR) was used to detect p53 mRNA level after treated with IL-7. Then using transmission electron microscopy (TEM) to observe the morphological change of autophagosome. 123 cases of NSCLC were collected for survival analysis, immunohistochemistry staining and cox regression multivariate analysis. Results We find that IL-7 induce the p53 translocation from nucleus to cytoplasm, then IL-7 down-regulate phosphorylation of AMPK and up-regulate phosphorylation of mTOR. The expression of AMPK and p53 were associated with IL-7/IL-7R and mTOR expression. Clinically, AMPK and p53 were well correlated with stage and survival of lung cancer patients. IL-7R, mTOR and tumor stage were the strongest predictors of survival. Conclusion In conclusion, IL-7 inhibit autophagy in NSCLC via P53 regulated AMPK/mTOR signaling pathway. AMPK and p53 are correlated with patients' survival. IL-7R, mTOR and tumor stage are the strongest predictor of survival.

Background

Over the years, Lung cancer still be the most threatening cancer in the world, for low survival rate(19%) and lack of effective screening method¹. Lung cancer are mainly classified into two types: non-small cell lung cancer(NSCLC) and small cell lung cancer(SCLC). Interleukin 7(IL-7), a member of IL-2 superfamily, is found to be essential in the development of B cell and T cell. Oddly, IL-7 seems play dual role in tumors. It is reported that IL-7 took anti-tumor effect in prostate cancer, glioma, melanoma, leukemia, and lymphoma. While IL-7 present pro-proliferation, promote lymphangiogenesis, and anti-apoptosis in NSCLC; increasing invasion and migration in bladder cancer cell; besides, breast cancer with higher grade and malignancy are associated with higher expression of IL-7²⁻⁵. Thus, the role of IL-7 in cancer are still elusive.

Autophagy (mainly represent for macroautophagy) is a self-eating process that transport the organelles and protein to autophagosome and fuses with lysosome. These protein can be degraded and then reused by cell for metabolism⁶. It is reported that cancer cell frequently observed with high level of autophagy which may essential for their proliferation and cope with stress⁷.

There are many autophagy related gene(ATG) and important signaling markers play a role in autophagy regulation. In our study, we will find out whether IL-7 regulate autophagy via p53, AMP-activated protein kinase (AMPK), mammalian target of rapamycin (mTOR) and light chain 3 (LC3, also known as mammalian homolog of Atg8)⁸ in NSCLC cell line.

Despite enormous study discuss about p53 play a role in autophagy. However the relationship between different status of p53 and autophagy are still elusive. It is reported that P53 activates autophagy and

autophagy suppress p53, they are functionally intertwined⁹. Many previous research showed that the localization of p53 appear to play dual role in autophagy. Cytoplasmic P53 inhibit autophagy, while nuclear p53 promote autophagy^{6, 10}. AMPK, a serine/threonine protein kinase, is composed of α, β, γ subunits and regulating energy status, such as glucose or lipid metabolism and mitochondrial biogenesis¹¹. Besides, AMPK promote autophagy via antagonize mTORC1 complexes and activate ULK1 complexes^{11, 12}. But which is the upstream factor between p53 and AMPK is not known. In our study, A549 (p53 wild type) cell line was employed to unveil whether IL-7 regulate autophagy via p53, AMPK and mTOR. In addition, we will explore whether AMPK, P53 expression correlated with with IL-7/IL-7R level, clinical stages, and NSCLC patient survival.

Material And Methods

cell culture and in vitro treatment

A549 cell line was purchased from Cell Bank of the China Academy of Sciences (Shanghai, China) and maintained in RPMI 1640(Gibco, Waltham, MA, USA). Media were supplemented with 10% fetal bovine serum (ExCell Bio, Shanghai, China, FSP500) and 100 U/ml penicillin(Sigma-Aldrich, St. Louis, MO, USA), 100 μ g/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Cell were cultured at 37°C in a humidified atmosphere containing 5% CO₂ until 80% confluent. Firstly, A549 cells incubated with IL-7(24 ng/ml) for different times at 0 h, 8 h and 12 h. Then A549 cell line were divided into several groups: control group; IL-7(24 ng/ml) stimulating 12 hours(12 h) group; anti-IL-7 receptor(A-IL-7R) group (cells incubated with A-IL-7R monoclonal antibody 100 ng/ml plus IL-7 24 ng/ml); p53 suppressing group: cells were incubated with pifithrin- α (PFT- α)(30 μ mol/ml) for 12 h; PFT- α and IL-7 group: cell preincubated with PFT- α for 2 hours then incubated with IL-7; AMPK inhibiting group: cell were incubated with Compound C(dorsomorphin)(30 μ mol/ml) for 12 h; and Compound C plus IL-7: cells pretreated with Compound C for 2 hours then treated with IL-7.

Reagents and antibody

IL-7 was purchased from Peprotech(Peprotech, Rocky Hill, NJ). P53(1:1000, Proteintech Group, Chicago, IL, USA), AMPK- α (1:1000, Wanlei Bio, Shenyang, China), β -actin(1:1000, Proteintech Group, Chicago, IL, USA), anti-mouse IgG (1:2000, Proteintech Group, Chicago, IL, USA) and anti-rabbit IgG(1:2000, Proteintech Group, Chicago, IL, USA), mTOR(1:1000, Cell Signaling Technology, Beverly, MA), phosphorylated(p)-mTOR(1:1000, Cell Signaling Technology, Beverly, MA) and p-AMPK(1:1000, Cell Signaling Technology, Beverly, MA), LC3B(1:1000, Cell Signaling Technology, Beverly, MA), anti-IL-7 receptor antibody(A-IL-7R) (Santa Cruz, CA), P53 inhibitor: PFT- α (MedChemExpress, Monmouth, NJ, USA) and AMPK inhibitor: Compound C (MedChemExpress, Monmouth, NJ, USA).

Western Blot Analysis

After IL-7 stimulation, total protein were extracted with NP-40 lysis buffer (Beyotime Biotechnology, Shanghai, China) (containing 1x protease and phosphatase inhibitors cocktail) on ice for 25 minutes. Then centrifuged at 12,000 rpm for 20 min at 4 °C. After quantified, total protein sample(40µg protein per lane) were electrophoresed on 8% and 12% SDS polyacrylamide electrophoresis (SDS-PAGE) gel then transferred to polyvinylidene difluoride(PVDF) membrane(Millipore, Billerica, MA, USA). Then, membrane were incubated with specific primary antibody in 4 °C overnight and secondary antibody for 2 h at room temperature. Finally, membrane were visualized using electrochemiluminescence (ECL) (Wanlei, Shenyang, China) and detected with BioImaging System (UVP Inc., Upland, CA, USA).

Cytoplasmic and nuclear protein were extracted by using cytoplasmic and nuclear protein extraction kit (Beyotime Biotechnology, Shanghai, China) according to manufacturer' s protocol. Nuclear protein(30 µg) and cytoplasmic protein(30 µg) were electrophoresed on 8% SDS-PAGE gel then transferred to PVDF membrane. Other process are similar as previously described.

RT-qPCR

Sample were isolated by trizol, then 1µg RNA were reverse transcribed into single-strand cDNA using PrimeScript RT reagent kit (Takara, Tokyo, Japan, RR047A). Finally SYBR Green Real Time PCR master mix (Takara, Tokyo, Japan, RR820A) was used to perform in 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Parameter of PCR reaction are as follows: incubation at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 60°C for 34 s, finally 95°C for 15 min, 60°C for 1 min and 95°C 15 s. Gene expression level were calculated by the $2^{-\Delta\Delta CT}$ method. Primer sequences are as follows:p53 forward: 5'-GCTGAGTATCTGGACGACAGG-3'; p53 reverse: 5'-AGCGTGATGATGGTAAGGATG-3', β-actin forward: 5'-ATAGCACAGCCTGGATAGCAACGTAC-3'; β-actin reverse: 5'-CACCTTCTACAATGAGCTGCGTGTG -3'.

Transmission Electron Microscopy

After A549 cell were treated corresponding to the experimental design described above, TEM was used to observe autophagosome in A549 cell. After cell were washed with phosphate buffer saline(PBS), they were digested with 0.25% trypsin and centrifugation in 1.5ml tube. Then sediment were fixed with glutaraldehyde for 2 h in 4°C. Subsequently, sample were post-fixed with osmium and dehydrated with ethanol. Finally, sample examined via TEM(Hitachi, Tokyo, Japan).

Patients and specimens

A total of 123 cases of NSCLC were obtained from the 1st January 1990 to the 31st December 2013 at General Hospital of Northern Theater Command, Shenyang, China. The tumour tissues in this study were from patients who had NSCLC proved by pathological diagnosis without distant metastasis. None of the 123 cases had received radiation therapy or chemotherapy before surgery. The TNM staging system of the UICC (2017) was used to classify the specimens.

The survival time was calculated from the operation day to death via the evaluation of recurrence and metastasis or until the last follow-up date (December 2013). The following-up of the surviving patients

averaged 27.09 months and ranged from 1 to 156 months. The study has been approved by the General Hospital of Northern Theater Command Ethics Committee.

Immunohistochemistry

Four-micron thick sections were prepared from the paraffin-embedded tissues. Immunostaining was performed by the streptavidin-peroxidase (S-P) method (Zhongshan, Beijing, China). The primary antibodies were anti-IL-7, anti-IL-7R, anti-P53, anti-AMPK and anti-mTOR (1:100, 1:100, 1:150, 1:100, 1:150) antibodies. The peroxidase reaction was developed with DAB. For negative control, the primary antibodies were replaced by non-immune serum.

All the samples were evaluated by two independent pathologists. The staining intensity and extent of IL-7, IL-7R, P53, AMPK, and mTOR were scored as follows. Staining intensity was defined as the depth of shade of the protein and graded as 0 (no staining), 1 (mild staining), 2 (moderate staining), 3 (strong staining). Staining extent was defined as the percentage of tumor cells with positive brown-yellow staining and graded as follows: 0 (< 10%), 1 (10%-49%), 2 ($\geq 50\%$). Then sum index of the two variables was obtained and a total score of 4 was considered as the point to categorize the staining as high (≥ 4) or low (< 4) ¹³.

Statistical analysis

SPSS19.0 (SPSS incorporated, Chicago) was used for all analysis. One-way analysis of variance(ANOVA) was performed for statistical analysis, and post hoc analysis Dunnett's test was used for each group to control. P-value < 0.05 was considered statistically significant. All the experiments were conducted in triplicate. The Chi-square test was used to analyze the relationship between P53 and AMPK expression and clinical pathological factors; these tests were also used to evaluate whether there was any significant relationship between P53/AMPK and IL-7/IL-7R; Kaplan–Meier curves were used for survival analysis, and log-rank was determined based on the differences; Cox regression multivariate analysis was used to evaluate P53 and AMPK as prognostic factors to compare them with other strong prognostic factors for prognosis in lung cancer.

Results

IL-7 induced the p53 translocation from nucleus to cytoplasm

We isolated nuclear and cytoplasmic protein of A549 at 8h and 12h. After IL-7 treated for 12h, we observed a notably increase of cytoplasmic p53(figure.1a), so as an escalation of p53 mRNA level(figure.1c). This effect could be blocked by anti-IL-7 receptor antibody(A-IL-7R)(figure.1b). These data suggest IL-7 affected p53 distribution between nuclear and cytoplasm in A549 cell line.

IL-7 affect phosphorylated form of AMPK and mTOR

We detected total protein of A549 cells after incubated with IL-7 and IL-7 plus its A-IL-7R antibody for 12 hours: the result show that p-AMPK decreased and p-mTOR increased, while total protein of AMPK and mTOR did not change, and this effect could be blocked by A-IL-7R, which indicate that IL-7 affect both p53, p-AMPK and p-mTOR (figure.2a).

IL-7 regulate mTOR via AMPK

As shown in (figure.3a), AMPK inhibitor Compound C shut down the p-AMPK/AMPK expression and escalate the p-mTOR/mTOR expression level. But the Compound C did not change the p53 expression neither nucleus nor cytoplasm (figure.3b).

IL-7 regulate AMPK and mTOR via p53

As shown in (figure. 4a), after PFT- α inhibit cytoplasmic and nuclear p53 level in A549, we observed an up-regulation of p-AMPK/AMPK and down-regulation of p-mTOR/mTOR(figure.4b). Furthermore, inhibition of p53 by PFT- α rescued IL-7-induced suppression of p-AMPK/AMPK and activation of p-mTOR/mTOR. On the contrary, for RT-qPCR results, PFT- α caused slight escalation of p53 mRNA expression level as shown in (figure.4c).

IL-7 inhibit autophagy of A549

To confirm that IL-7 regulate autophagy suppression, western blot were used to analyze LC3-II/I protein level and TEM were applied to observe autophagosome as the group previously designed: negative control group, cells treated with IL-7, PFT- α , IL-7 + PFT- α , Compound C, Compound C + IL-7, and IL-7 + A-IL-7R. Western blot for LC3 show that IL-7 decrease LC3-II/I protein level and this can be blocked by A-IL-7R. In addition, PFT- α increase the LC3-II/I and Compound C decrease the LC3-II/I level(Fig. 2b, Fig. 3c, Fig. 4d). Through the TEM assay, we further observed the change of autophagosome for each group: IL-7 group, Compound C group and IL-7 + Compound C group find out a decrease of autophagosome; PFT- α and IL-7 + PFT- α group observed a noteworthy increase of autophagosome; control group observed an basic autophagosome level in A549 cells and the autophagosome level of IL-7 + A-IL-7R group is similar to control group (figure.5). Both the LC3 and TEM results indicate IL-7 inhibit autophagy of A549 cell line.

Expression of AMPK and P53 correlate with IL-7/IL-7R level, clinical stages, and NSCLC patient survival

Immunohistochemical analysis of 123 NSCLC specimens revealed that the frequency of high expression of AMPK was 39.84%(49/123). The high expression of AMPK was significantly associated with the low expression of IL-7, IL-7R, mTOR, and P53 (Table 1, Fig. 6). Clinically, compared to the NSCLC with high expression of AMPK, tumors with low expressions of AMPK were more advanced-stage cancer ($P < 0.001$). Patients with high expression of AMPK (median survival = 63 ± 24.92 months; 95% confidence interval 14.157–111.843 months) had a statistically significant longer survival than those with low expression of AMPK (median survival = 16 ± 2.608 months; 95% confidence interval 10.888–21.112 months; $P < 0.001$; Fig. 7a).

Table 1
Relationship between AMPK expression in NSCLC and clinical pathological factors (Chi-square test)

	Patients	AMPK high expression(%)	P value	χ ² value
Gender				
Male	89	35(39.33%)	1.000	0.035
Female	34	14(41.18%)		
Age				
≤ 60	71	30(42.25%)	0.579	0.409
> 60	52	19(36.54%)		
Histology				
Squamous cancer	72	27(37.5%)	0.578	0.396
Adenocarcinoma	51	22(43.14%)		
Differentiation				
Well	16	6(37.5%)	0.739	0.604
Moderate	55	24(43.64%)		
Poor	52	19(36.54%)		
Stage				
I-II	56	32(57.14%)	0.000	12.846
III	67	17(25.37%)		
mTOR expression				
high	72	16(22.22%)	0.000	22.481
low	51	33(64.71%)		
P53 expression				
high	71	19(26.76%)	0.001	11.983
low	52	30(57.69%)		
IL-7 expression				
high	89	24 (26.97%)	0.000	22.255
low	34	25(73.535%)		

	Patients	AMPK high expression(%)	P value	χ^2 value
IL-7R expression				
high	81	28 (34.58%)	0.000	19.154
low	42	21 (50%)		

However, the frequency of high expression of P53 was 57.72%(71/123). The high expression of P53 was significantly associated with the high expression of IL-7, IL-7R, mTOR, and AMPK low expression(Table 2, Fig. 6). The high expression level of P53 was significantly associated with clinical stage of tumors($P = 0.01$). In addition, patients with low expression of P53 (median survival = 30 ± 22.5 months; 95% confidence interval 0–74.1 months) survived longer than those with high expression of P53 (median survival = 13 ± 2.174 months; 95% confidence interval 8.74–17.26 months; $P < 0.001$; Fig. 7b).

Table 2
Relationship between P53 expression in NSCLC and clinical pathological factors
(Chi-square test)

	Patients	P53 high expression(%)	P value	χ ² value
Gender				
Male	89	47(52.81%)	0.102	3.187
Female	34	24(70.59%)		
Age				
≤ 60	71	41(57.75%)	1.000	0.000
> 60	52	30(57.69%)		
Histology				
Squamous cancer	72	41(56.94%)	0.855	0.043
Adenocarcinoma	51	30(58.82%)		
Differentiation				
Well	16	9(56.25%)	0.899	0.212
Moderate	55	33(60%)		
Poor	52	29(55.77%)		
Stage				
Ⅰ-Ⅱ	56	25(44.64%)	0.01	7.208
Ⅲ	67	46(68.66%)		
mTOR expression				
high	72	51(70.83%)	0.001	12.229
low	51	20(39.22%)		
AMPK expression				
high	49	19(38.78%)	0.001	11.983
low	74	52(70.27%)		
IL-7 expression				
high	89	62(69.66%)	0.000	18.807
low	34	9(26.47%)		

	Patients	P53	P value	χ^2
		high expression(%)		value
IL-7R expression				
high	81	58(71.6%)		
low	42	13(30.95%)	0.000	18.731

The expression levels of P53 and AMPK protein were not associated with a patient's age or sex, the tumor histological type, and differentiation (Table 1,2).In the Cox regression multivariate analysis, IL-7R, mTOR, and tumour stage were the strongest predictors of survival (Table 3)

Table 3
Multivariate Cox regression mode

	Wald	Sig.	Exp(B)	95% CI for Exp(B)
sex	0.066	0.798	0.932	0.546–1.591
age	0.136	0.713	0.919	0.587–1.439
histology	0.109	0.741	1.084	0.672–1.748
differen	1.918	0.383		
differen(1)	0.262	0.609	0.826	0.398–1.716
differen(2)	1.917	0.166	0.705	0.431–1.156
stage	20.685	0.000*	0.296	0.175-0.500
IL-7	0.521	0.47	0.711	0.281–1.796
IL-7R	14.582	0.000*	0.160	0.062–0.409
mTOR	4.076	0.043*	0.539	0.296–0.982
P53	0.611	0.434	1.236	0.726–2.104
AMPK	0.054	0.816	0.932	0.516–1.684

Discussion

Autophagy plays dual role in cellular activities. On the one hand, by degrading and reusing damaged cellular organelles, autophagy maintain cellular survival and metabolism⁶. Some cancer cell lines, such as pancreatic cancer cells, have abnormally high autophagy level⁷. It is likely that autophagy is required for cancer aggression¹⁴. On the other hand, BECN1(Beclin-1), an autophagy associated gene which play a

role in autophagosome formation, are frequently deleted in some cancer, such as breast cancer, ovarian and prostate cancer¹⁵. Other autophagy proteins are observed aberration and expression alteration. There is an emerging hypothesis that autophagy may act as anticarcinogenesis in the beginning status of oncocytoma¹⁵, but once tumor is established, autophagy may increase for cancer cell survival and fight against stress^{15,16}.

The role of IL-7 in tumor apoptosis, migration, proliferation and lymphangiogenesis has been reported. But its role in autophagy are still elusive. In a recent study, Jifeng Zhu etc revealed that IL-7 inhibit macroautophagy in schistosoma infected mice and this effect of IL-7 may exacerbated liver pathology¹⁷. In our latest study, we find IL-7 suppress formation of autophagosome in NSCLC, mainly by regulating AMPK/mTOR signaling pathway via translocation of nuclear/cytoplasmic p53 and regulating PI3K/AKT/mTOR signaling pathway via Beclin-1¹⁸. In addition, the effect of IL-7 could be blocked by A-IL-7R antibody.

Autophagy or glucose restriction leads to p53 degradation or suppression, but this effect are associated with p53 status^{9,19}. Interestingly, there is also evidence suggest that p53 activate autophagy, for p53 directly or indirectly targeting autophagy related gene such as DRAM, ULK1 and Atg7^{9,19}. Cytoplasmic p53 inhibit autophagy while nuclear p53 promote autophagy⁶. But the mechanism of how does different status of p53 affect autophagy are not known. In our study, A549 cell line(p53 wild type) were chose. Previously, we reported that IL-7 regulate autophagy by PI3K/AKT/mTOR signaling pathway via Beclin-1¹⁸ and prevent apoptosis via p53²⁰. IL-7 caused up-regulation of p-mTOR and change of p53. Thus, we asked whether p53 get involved in IL-7 regulated mTOR pathway and autophagy. By isolation of cytoplasmic and nuclear protein of p53 we find IL-7 cause p53 translocation from nucleus to cytoplasm at 12 hour, we also observed an increase of p53 mRNA level by using RT-qPCR. Then, by detecting total protein of AMPK and mTOR and its phosphorylated form, we observed IL-7 caused decrease of p-AMPK and increase of p-mTOR.

Mechanistically, several molecular, such as AMPK, DRAM, DAPK-1, PTEN, IGF-BP3 have been reported as downstream factor of p53²¹. It is reported that some kinases, including CAMKK, LKB1, TGF- β -activated protein kinase 1 (TAK1), and ataxia telangiectasia mutated (ATM) kinase play a role as upstream factor of AMPK²². However, whether p53 regulate AMPK or AMPK affect p53 are still elusive²³. Thus, p53 inhibitor PFT- α and AMPK inhibitor Compound C was used to reveal the relationship between p53 and AMPK. After incubated with PFT- α , western blot results show that both nuclear and cytoplasmic p53 protein decreased, then we observed increase of p-AMPK expression level and decrease of p-mTOR level. Meanwhile, PFT- α block the effect of IL-7-regulated autophagy suppression. Compound C could up-regulate p-mTOR expression and inhibit autophagy, but p53 protein level was not affected. These results demonstrated that p53 is the upstream molecular of AMPK, however, AMPK is not the upstream factor of p53 in IL-7 regulated autophagy in A549 cell line.

LC3, the mammalian homologue of yeast Atg8, is localized in autophagosome membrane and commonly considered as autophagy marker²⁴. But sometimes an increase in autophagosome does not correlate with LC3 aggregate²⁵. Thus TEM is another reliable way to assess autophagy level. In our work, both LC3 protein level and TEM results indicate that IL-7 inhibit autophagy in A549 cell, and these effect can be blocked by A-IL-7R. In addition, PFT- α notably activate autophagy demonstrating that p53 suppressing lead to autophagy activation. Besides, AMPK inhibiting cause autophagy suppression.

In our study, immunohistochemical analysis of 123 NSCLC specimens show that AMPK low expression are associated with p53, mTOR, IL-7, IL-7R high expression. Clinically, patients with AMPK low expression are more associated with advanced-stage cancer($P < 0.001$) and patients with high expression of AMPK had a statically significant longer survival than those with low expression of AMPK. Similar to our findings, Nan Li et al. find out in both squamous cell carcinoma and lung adenocarcinoma, p-AMPK low expression had a higher survival²⁶. However, Daohui Gong et al. demonstrated that AMPK α 1 was highly expressed in NSCLC cancer tissues and correlated with poor prognosis in patients with NSCLC²⁷. The immunohistochemical analysis of p53 showed the opposite results with AMPK. Zhihua Teng et al believe that lung cancer with p53-negative expression had a longer 3-year survival rate than those with p53-positive expression²⁸. Cox regression multivariate analysis show IL-7R, mTOR, and tumor stage were independent predictors of survival.

In our present study, we have illustrated basic mechanism of IL-7-regulated autophagy inhibition. However, whether IL-7-regulated autophagy pro-apoptosis or anti-apoptosis in NSCLC are not known. Thus, Further studies should be performed to this issue.

In conclusion, we demonstrated that IL-7 induced p53 translocate from nucleus to cytoplasm then regulating AMPK/mTOR signaling pathway to inhibit autophagy. Expression of AMPK and P53 correlate with IL-7/IL-7R level, clinical stages, and NSCLC patient survival. Targeting mTOR might be another therapeutic way for IL-7 regulated autophagy in NSCLC.

Abbreviations

Abbreviation	Definition
IL-7	Interleukin 7
AMPK	AMP-activated protein kinase
mTOR	mammalian target of rapamycin
LC3	Light chain 3
RT-qPCR	Real-Time quantitative PCR
TEM	transmission electron microscopy
NSCLC	Non-small cell lung cancer
SCLC	small cell lung cancer
ATG	autophagy related gene
PFT- α	pifithrin- α
A-IL-7R	Anti-IL-7 receptor antibody
SDS-PAGE	SDS polyacrylamide electrophoresis
PVDF	Polyvinylidene difluoride
ECL	Electrochemiluminescence
PBS	Phosphate Buffer Saline
NC	Negative Control
CC	Compound C

Declarations

Acknowledgements

Not applicable

Authors' contribution

JM and YZ designed the study. YZ performed the experiment; collected and analyzed the data; drafted the manuscript. ZD and XJ collected partial data. JM supervised the entire project, data analysis and writing of the manuscript. All authors have reviewed and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The data that support the findings of the present study are available from the corresponding author on reasonable request.

Approval for human experiments

1.Our study was approved by General Hospital of Northern Theater Command Ethics Committee.
2.Confirms that all experiments were performed in accordance with relevant named guidelines and regulations. 3.Written informed consent was exempted by Northern Theater Command Ethics Committee because we used retrospective sample and data, and our study obey the ethics regulation.

Ethics Approval

The study has been approved by the General Hospital of Northern Theater Command Ethics Committee.

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Figures

Figure 1

Isolation of cytoplasmic and nuclear p53 protein for A549 cells after treated with interleukin 7 (IL-7), anti-IL-7 receptor antibody (A-IL-7R) for western blot analysis. Then total RNA was isolated for real-time quantitative PCR(RT-qPCR) assay after incubated with IL-7 for different time. (a) A549 cells were treated with IL-7 at 24 ng/ml for 8 h and 12 h , then p53 nuclear and cytoplasmic protein were detected. Lamin B was used as nuclear loading control and β -actin was used for cytoplasmic loading control. Bar show the cytoplasmic and nuclear p53 western blot analysis. (b)A549 cells were treated with IL-7 and A-IL-7R plus IL-7 for 12 hours, then nuclear and cytoplasmic p53 protein were detected by western blot. Bar show the cytoplasmic and nuclear p53 western blot analysis. (c) After incubated with IL-7 at different time (0h, 8h, 12h), p53 mRNA level was detected by RT-qPCR, all the bar graph expressed as Mean $\pm$ SD, n = 3. *represent for P < 0.05 vs. negative control(NC). **represent for P < 0.01 vs. NC.

Figure 2

IL-7 regulating the expression of AMPK/mTOR pathway and inhibit autophagy. (a) Western blot analysis showing down-regulation of phosphorylated (p)-AMPK proteins and up-regulation of p-mTOR protein in A549 cells after treated with IL-7, and using A-IL-7R specific antibody block the effect of IL-7, however the expression level of total AMPK and mTOR are not significantly affected. (b) Cell treated with IL-7 lead to LC3II/I decrease, and this effect can be blocked by A-IL-7R. P-AMPK/AMPK, p-mTOR/mTOR and LC3II/I results are expressed as Mean $\pm$ SD, n = 3. *represent for P < 0.05 vs. NC.

Figure 3

Cells were treated with IL-7 and Compound C to demonstrate that AMPK is upstream factor of mTOR. (a) IL-7 and Compound C both cause the down-regulation of p-AMPK/AMPK and up-regulation of p-mTOR/mTOR in A549 cells. (b) Isolation of p53 cytoplasm and nucleus protein. Compound C did not cause p53 expression change neither nuclear nor cytoplasmic. While IL-7 caused the expression change in cytoplasmic p53. (c) Western blot analysis for LC3 α/β as group designed above. The bar graph corresponding to their western blot results show different kind of protein analysis after cell treated with IL-7, Compound C and IL-7+Compound C, then compared with negative control. Mean $\pm$ SD, n = 3. *represent for P < 0.05. ** and *** represent for P < 0.01 vs. NC.

Figure 4

Cells were treated with IL-7 and pifithrin- α (PFT- α) to demonstrate that P53 is the upstream factor of AMPK and mTOR. (a) PFT- α suppress p53 expression both in cytoplasmic and nuclear. IL-7 mainly cause cytoplasmic p53 increase. (b) In contrast to IL-7 effect in A549 cells, PFT- α caused increase of p-AMPK/AMPK and decrease of p-mTOR/mTOR. (c) Real-time PCR assay for p53 mRNA analysis as group designed above. Both IL-7 and PFT- α cause p53 mRNA level increase. (d) The LC3B-II/I western blot analysis for PFT- α , IL-7 and IL-7+PFT- α vs control group. PFT- α notably increase LC3B-II/I level. Bar graph corresponding to their assay further expressed the p53, P-AMPK, P-mTOR and LC3B-II/I protein analysis as well as p53 mRNA analysis. Mean $\pm$ SD, n = 3. *represent for P < 0.05 vs. NC. ** and ***represent for P < 0.01 vs. NC.

Figure 5

Transmission Electron Microscopy (TEM) results show the group as designed above. Images were taken at either 8000x or 20000x. The 20000x image was the enlarged image of 8000x in the black frame. In the 20000x, the black arrow indicate characteristic autophagosome in A549 cells after treated with IL-7 for 12 h (autophagosome decrease); cells treated with pifithrin-α for 12 h (autophagosome notably increase); cells treated with pifithrin-α+ IL-7 for 12 h (autophagosome increase); cells treated with Compound C for

12 h (autophagosome decrease); cells incubated with Compound C + IL-7 (autophagosome decrease); and cells treated with A-IL-7R antibody + IL-7 (effect of IL-7-induced autophagosome depression was blunt).

Figure 6

In both adenocarcinoma and squamous-cell carcinoma, AMPK low expression correlates with high expression of P53, mTOR, IL-7 and IL-7R. Meanwhile, The high expression of P53 was significantly associated with the high expression of IL-7, IL-7R, mTOR, and AMPK low expression.

Figure 7

Expression of AMPK and p53 correlates with survival of NSCLC patients. (a) AMPK high expression had a longer survival than those with AMPK low expression. (b) P53 low expression had a longer survival

compared with P53 high expression.

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