Pakistan J. Zool., pp 1-8, 2023. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20221115081110

LY6K Promotes Proliferation and Energy Metabolism of Lung Adenocarcinoma Cells by Regulating Aerobic Glycolysis

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Article Information
Received 15 November 2022
Revised 20 December 2022
Accepted 19 January 2023
Available online 13 May 2023
(early access)

Authors' Contribution
Conception and design of the work: WY and LL. Data collection: LN, ZYJ, LW, XWH, WQ, ZPJ, FHL. Supervision: WY and LL. Analysis and interpretation of the data: WY, LL, LN, ZYJ, LW, XWH, WQ, ZPJ, FHL. Statistical analysis: WY, LL, FHL. Drafting the manuscript: WY and LL. Critical revision of the manuscript: WY, LL, FHL. Approval of the final manuscript: all authors.

Key words
LY6K, Lung adenocarcinoma cell, Aerobic glycolysis, Cell proliferation, Energy metabolism

ABSTRACT

The main objective of this study was to investigate the effects of lymphocyte antigen 6-complex K (LY6K) on aerobic glycolysis and proliferation of lung adenocarcinoma cells. Database analysis confirmed the association between LY6K expression and survival prognosis. Western blot was used to detect the protein expression of proteins. RT-PCR was used to detect the mRNA expression level of genes. Cell proliferation was discovered using the CCK8 test and plate cloning. The impact on the cell cycle was examined by using flow cytometry. The production of glycolytic enzymes and the significance of LY6K in the development of lung cancer in naked mice were both noted. The effects of LY6K knockdown on glucose uptake rate, lactic acid and energy metabolism of A549 cells were determined by UV spectrophotometry. According to data analysis from GEPIA2, TCGA, and Kaplan Meier plotter, lung adenocarcinoma exhibits high levels of LY6K expression and is associated with a poor prognosis. The expression of LY6K was most significant in A549 cells. In A549 cells, LY6K knockdown significantly inhibited cell proliferation and plate clonal formation (all $p < 0.05$), and inhibited tumor formation in nude mice ($p < 0.01$). The protein expression levels of GLUT1, HK2, PFKL, ALDOA, PGK-1, PKM2 and LDHA were down-regulated (all $p < 0.05$), and the glucose consumption and the contents of lactic acid and ATP were decreased (all $p<0.05$). To conclusion by regulating the production of enzymes involved in aerobic glycolysis, LY6K may encourage the proliferation and energy metabolism of A549 cells.

INTRODUCTION

According to Bray *et al*. (2018), lung cancer has the highest incidence and fatality rate of all malignant tumors and is increasing annually. Lung adenocarcinoma is the most prevalent subtype of non-small cell lung cancer (NSCLC), which accounts for around 85% of all instances of lung cancer. The two leading causes of mortality in lung cancer patients are metastasis and tumor recurrence. The molecular basis for the emergence and progression of lung cancer must thus be well understood. Studies have shown that one of the most distinguishing characteristics of tumor cells is aberrant metabolism, which serves as the molecular underpinning for the fast proliferative and metastatic spread of tumor cells (Park *et al*., 2020). Regardless of aerobic conditions or anaerobic conditions, tumor cells would preferably adopt glycolysis to obtain the energy needed by cells (Van der Heiden *et al*., 2009). Therefore, it is concluded that the process of glycolysis can be inhibited by inhibiting the activity of glycolytis-related enzymes in

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0030-9923/2023/0001-0001 $ 9.00/0

tumor cells, thus inhibiting the proliferation of tumor cells (Cozzo *et al*., 2020; Moldogazieva *et al*., 2020).

A protein superfamily known as lymphocyte antigen 6 (Ly-6) or Ly-6/uPAR is made up of eight or ten cysteine residues that create four or five conserved disulfide bonds in a conserved LU domain. The prognosis of patients is inversely associated with the aberrant expression of several members of the Ly6/uPAR superfamily in cancer. In addition, the expression of Ly6/uPAR superfamily members in immune cells has been confirmed (Upadhyay, 2019; Lee *et al*., 2013). The roles of the majority of family members are still unclear despite these advancements. The LY6K gene is one member of the LY6/u PAR superfamily. Normal cells only express LY6K locally in testicular germ cells, which may encourage sperm cell migration. LY6K is overexpressed in a variety of human malignant tumors, while its expression is low or difficult to detect in the corresponding normal tissues (Suzuki *et al*., 2013; Al-Hossiny *et al*., 2016; Choi *et al*., 2009; Luo *et al*., 2016; Matsuda *et al*., 2011; Ishikawa *et al*., 2007). Overexpression of LY6K is closely associated with aggressive growth and increased activity as well as poor prognosis and recurrence of several tumor types. However, it has not been proved whether LY6K can participate in the carcinogenesis process of lung adenocarcinoma by regulating the glucose metabolism of cancer cells. As a result, this research used genes associated with the glycolytic pathway as its starting point to investigate the precise mechanism by which LY6K affects lung cancer.

MATERIALS AND METHODS

The expression of LY6K mRNA in lung cancer tissues and healthy tissues was examined using the GEPIA2, TCGA, and Kaplan Meier plotter databases.

Cell culture and grouping

A549 cells were obtained from Peking Union Medical College Cell Resource Center, H1299 cells and H358 cells were purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, and human lung bronchial epithelial cell BEAS-2B cells were obtained from Kunming Cell Bank, Chinese Academy of Sciences. Incubated at 37°C in a 5% CO2 incubator, A549 cells were grown in RPMI-1640 (Gibco) medium with 10% fetal bovine serum and BEAS-2B cells in DMEM high-glucose media (Gibco) with 10% fetal bovine serum. Cells in the logarithmic development stage were chosen for the experiment, and passages were carried out every one to two days. Negative control and LY6K siRNAs, designated si-NC and si-LY6K, respectively, were transfected into A549 cells. Cell transfection was carried out using Lipofectamine 2000 (Invitrogen) transfection reagent. Please refer to the transfection reagent's instructions for the technique.

Real-time quantitative PCR (qRT-PCR)

Cell samples were taken after 48 h of growth in 6-well plates using cells from various transfection groups. Utilizing an RNA extraction kit (Promega), total RNA was extracted, its quantity measured, and then reverse-transcribed into cDNA. For quantitative PCR analysis utilizing the dye technique (SYBR Green I), the reaction system was put in the fluorescence quantitative PCR system, and β-actin was employed as the internal reference. The primer sequence is presented in Table I for the primers used in this experiment, which were created and synthesized by Shanghai Sangong Company.

Table I. Primers used in real-time PCR.

Genes	Primers 5'→3'
LY6K	F:5'-CTGACTGCGAGACAACGAGAT-3'
	R:5'- ATTTGCACCTCCTTGGGTTCT-3'
GLUT1	F:5'-ACATAGCTTGCCTAATGGCTTTCAC-3'
	R:5'- CCTGCCTGCTGACAACACCTA-3'
HK2	F:5'-GCAGCGCATCAAGGAGAACAAAG-3'
	R:5'-GGAGCGGAGGAAGCGGACAT-3'
PFKL	F:5'-CATCGCTGAGGGTGCCA-3'
	R:5'-AACCACCAGGTCCTTCACG-3'
AL-DOA	F:5'-CCCAAGCTTATGCCCTACCAATATCCAGCAC-3'
	R:5'-CGGAATTCTTAATAGGCGTGGTTAGAGAC-3'
PGK-1	F:5'- TCACTCGGGCTAAGCAGATT-3'
	R:5'-CAGTGCTCACATGGCTGACT-3'
PKM2	F:5'-CTGGGGCTGCTGTGGACTTG-3'
	R:5'- AGATGCCTTGCGGATGAATGAC-3'
LDHA	F:5'-CAACATGGCAGCCTTTTCCTTAGA-3'
	R:5'-ATCCAGATTGCAACCGCTTCC-3'
β-actin	F:5'-GAGCTACGAGCTGCCTGACG-3'
	R:5'-GTAGTTTCGTGGATGCCACAG-3'

Western blot

The total protein in the cells was extracted after the two cell groups had been incubated for 48 h, and the protein was then quantitatively quantified using the BCA (Sigma-Aldrich) technique. For electrophoresis, 40 μg of protein samples from each group were introduced, along with concentration glue at 90 V and separation glue at 120 V. To transfer the proteins from the gel to the nitrate cellulose membrane, the membrane was moved at 190 m A. The appropriate primary and secondary antibodies were

incubated with membranes using 5% skim milk for 2 h and next the results were recorded using the chemiluminescence technique. The information of primary antibodies are as follows: LY6K antibody (ab246486, Abcam), GLUT1 antibody (21829-1-AP, Proteintech), HK2 antibody (#2867, Cell Signaling Technology, USA), PFKL antibody (ab97443, Abcam, USA), ALDOA antibody (ab252953, USA) Abcam), PGK1 antibody (#68540 cell signaling technology), PKM2 antibody (#4053 cell signaling technology), LDHA antibody (#3582, cell signaling technology, β-actin (#3700, cell signaling technology), CyclinD1 antibody (#55506, cell signaling technology), CDK4 antibody (#23972, cell signaling technology, USA). For each group, the experiment was conducted three times.

CCK8 assay

A549 cells from the si-NC group and si-LY6K group were incubated onto 96-well plates for 1×10^4 cells per well during the logarithmic growth stage. Each well received 10 μL of the CCK8 solution after being grown for 24 h, 48 h, and 72 h, and was then given another hour of cell incubator growth. Each well's OD value was discovered at 450 nm wavelength.

Plate clone formation experiment

The two groups' transfected cells were infused onto 6-well plates with 1000 cells per well (each group had 3 multiple wells). For 7 to 14 days, the cells were cultivated at 37 °C in a 5% CO2 incubator. Each day, the cells' condition was checked, and the necessary fluid was replaced. When the single cell clonal colony reached a size of roughly 50 cells per colony or became visible to the naked eye, the culture was stopped. The pore plate cells were fixed for 30 min in 10% formaldehyde, then stained for 10 min in 1% crystal violet. The PBS was used to gently clean the crystal violet dye solution. It was examined and counted how big each colony was in the orifice plate. It was determined what each numerous orifice's average value was.

Cell cycle

After si-NC and si-LY6K were transfected into A549 cells, the cells were removed 48 h later, washed twice with PBS, and then re-suspended. The supernatant was then removed, a solution of propyl iodide (PI) was added, and the cells were stained for 30 min away from light. Then the machine (FACSAria ™ III flow cytometer, BD, USA) picked up the cells. The program Flow Jo 7.6 was used to analyze the data.

Tumor bearing experiment in nude mice

Laboratory animal

Twelve female athymic nude mice (BALA/c-nu) were purchased from Beijing Weitong Lihua Laboratory Animal Technology Co., Ltd. In an SPF sterile laminar flow environment with a temperature of 26–28°C and a humidity of 40%–60%, twelve BALB/c female nude mice were raised for 5 weeks. With a light cycle of 10 h of sunshine and 14 h of light shelter, 6 mice per cage were fed twice daily. The Hebei University of Traditional Chinese Medicine's Animal Experiments Committee gave its approval to the experimental methods and procedures, which were carried out in compliance with the ethical guidelines for using animals in research.

Subcutaneous inoculation (proliferation model)

Twelve nude mice were separated into two groups at random: Six were placed in the si-NC group and six were placed in the si-LY6K group. A549-siLY6K cells and control cells were grown *in vitro* and injected into the subcutaneous region of the right axilla of naked mice using 100 μL of 1 x 10^7 cells per cell. Every three days when the tumor was palpable, its size was measured using a vernier caliper. To plot the tumor development curve for the two groups of animals, the tumor volume was computed using the formula:

Tumor volume = (length × width ^{2}) x 1/2.

To examine the impact of LY6K knockdown on tumor development, the tumor was excised under anesthesia after 6 weeks after inoculation, documented, and weighed.

Expression of LY6K and glycolytic related enzymes in tumor-bearing tissue of nude mice

The total protein in the tumor tissue was extracted, and Western blot analysis was used to determine how LY6K knockdown affected the expression of glycolytis-related enzymes. These are the precise steps: The 400 μl cell lysate (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, and 1% Triton X-100, and placed into cocktail before use) was used to thoroughly homogenize and the ice lysate for one hour after the tumor tissue was frozen to the size of a mung bean. The protein solution was centrifuged at 12000 rpm for 30 min at 4°C after being intermittently oscillated for 10 min. Carefully absorbed, the supernatant was then kept at -80°C for future use. Before using the protein concentration in clinical settings, it was measured using the BCA protein quantification kit.

Detection of glucose, lactic acid and ATP levels

A549 cells were cultivated at a density of 80%–90% in a favorable growth stage. According to the experimental groups, cells were seeded onto 6-well plates, and when the cell densities in each group reached roughly 70%, cells were transfected. Following the addition of the transfection reagent, the transfection reagent was grown in

the cell incubator for 24, 48, and 72 h at 37°C, 5% CO_2, and saturated humidity. The glucose determination kit, lactic acid kit, and ATP kit were used to measure the glucose level, lactic acid generation, and ATP concentration at three distinct time intervals, in accordance with the kit instructions (24h, 48h and 72h). Glucose detection kit, lactate, pyruvate content kit and ATP kit were purchased from Jiancheng Bioengineering Institute, Nanjing, China.

Statistics analysis

Data were presented as mean±standard deviation (SD). GraphPad Prism software was used to run the T-test, one-way analysis of variance (ANOVA), and two-factor ANOVA. The statistical program SPSS 22.0 was used to evaluate the chi-square test, and a difference of $p<0.05$ was deemed significant.

RESULT

The expression of LY6K in lung adenocarcinoma was verified by the database

The GEPIA2, TCGA, and Kaplan Meier plotter databases confirmed the expression differential of LY6K and its connection to survival and prognosis. The findings demonstrated that lung adenocarcinoma tissues had considerably greater levels of LY6K mRNA expression than did healthy lung tissues (Fig. 1A, B). According to Kaplan-Meier analysis (Kaplan Meier plotter: Log-rank test P = 0.0024, Fig. 1C; GEPIA2: P = 0.0024; TCGA: P = 0.0067, Fig. 1E), the overall survival rate of the high LY6K expression group was substantially lower than that of the low LY6K expression group. The findings demonstrated that lung cancer had high levels of LY6K expression and was linked with a poor prognosis.

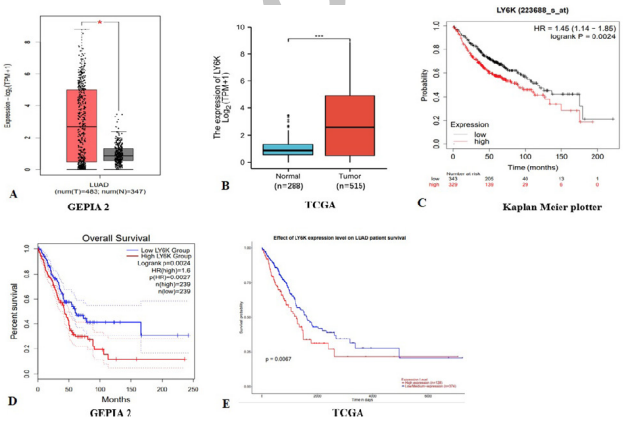

Fig. 1. Expression of LY6K gene in lung adenocarcinoma. A-B, Expression of LY6K mRNA in GEPLA2 and TCGA databases. C-E, Survival analysis of LY6K mRNA in Kaplan Meier plotter, GEPLA2 and TCGA databases.

Expression of LY6K in cells

Then, it was investigated how LY6K was expressed differently in adenocarcinoma cells (H1299, A549, and H358) compared to normal lung epithelial BEAS-2b cells. Results from a western blot revealed that A549 cells had the highest levels of LY6K expression in comparison to the control group's BEAS-2b cells (Fig. 2A, B). As a result, A549 cells became the primary study subject.

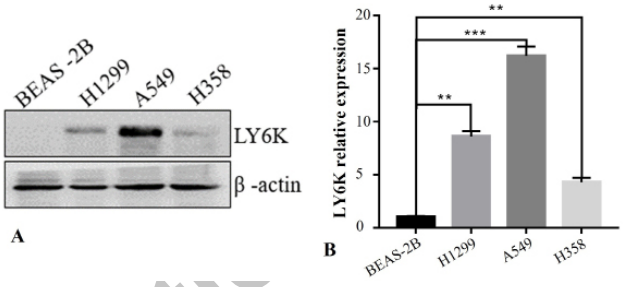

Fig. 2. Protein expression of LY6K in lung cancer cells. A, Protein expression of LY6K in lung cancer cells. B, protein quantification in A. **p <0.01; ***p <0.001.

Cell transfection efficiency

Following transfection of si-LY6K, the mRNA and protein levels of LY6K in A549 cells dramatically reduced as compared to the control group, according to the findings of qRT-PCR and Western blot. ($p<0.01$, Fig. 3).

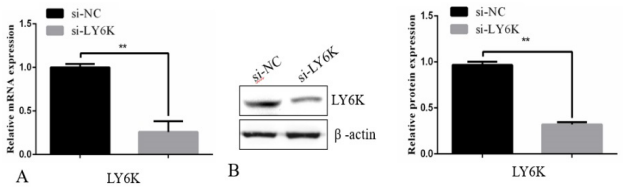

Fig. 3. Transfection efficiency. A, Expression of LY6K mRNA in A549 cells after transfection of si-LY6K, **p <0.01; B, Expression of LY6K protein in A549 cells after transfection of si-LY6K, ** p <0.01.

Effect of LY6K on proliferation of A549 cells

CCK-8 experiment showed that at 48 and 72 h, the OD value of the si-LY6K group of A549 cells was considerably lower than si-NC ($p<0.05$, Fig. 4A), while at 24 h there was no significant difference.

Plate cloning experiment showed that in the transfected si-NC group, the number of cell clones greatly increased after 7 days of cell culture, but the number of cell clones in the transfected si-LY6K group dramatically reduced ($p<0.01$, Fig. 4B). The results demonstrated that LY6K knockdown drastically reduced A549 cells' capacity to generate plate-to-plate clones.

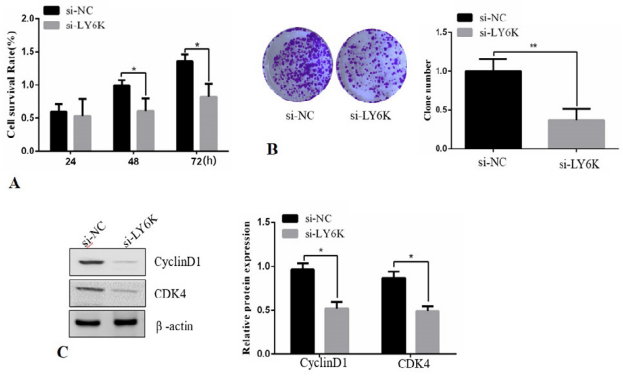

Fig. 4. Effect of LY6K on proliferation of A549 cells. A, Effect of LY6K knockdown on proliferative activity of A549 cells, * p <0.05; B, Effect of LY6K knockdown on A549 cell cloning, ** p <0.01; C, Effects of LY6K knockdown on CyclinD1 and CDK4 in A549 cells, * p <0.05.

FCM cell cycle assay showed that 48 h after transfection with the si-LY6K group, the percentage of A549 cells in the G0/G1 phase was considerably higher (p< 0.01, Table II) compared to the si-NC group, while the proportion of cells in the S and G2/M phases was lower (p<0.05, Table II). According to the findings, LY6K knockdown may cause A549 cells to stall in the G1 phase and prolong the cell cycle.

Table II. Effects of LY6K on cell cycle distribution of A549 (%, $\bar{x}$ ±SD).

Groups	G0/G1	S	G2/M
si-NC group	49.54±0.90	27.53±1.67	22.93±0.91
si-LY6K group	62.72±1.40**	20.13±0.91*	17.15±1.86*

Note: Compared with the si-NC group,** P<0.01,* P<0.05

Western blot results showed that CyclinD1 and CDK4 expressions were lower in the si-LY6K group than in the si-NC group (p <0.05, Fig. 4C).

LY6K knockdown decreased the expression of glycolytic-related enzymes in A549 cells

After transfection for 48 h, the experiment was split into a negative control group (si-NC) and an experimental group (si-LY6K) to better understand the effect of LY6K on the expression of enzymes involved in the glycolysis process. In comparison to the si-NC group, the expression of GLUT1, HK2, PFKL, ALDOA, PGK-1, PKM2, and LDHA was dramatically downregulated in A549 cells following transfection of si-LY6K, according to the findings of qRT-PCR and western blot (p <0.05, Fig. 5).

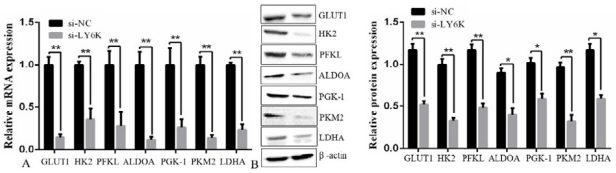

Fig. 5. The effect of LY6K transfection on the expression of key glycolytic enzymes. A, The mRNA expression level of glycolytic related enzyme after LY6K knockdown in A549 cells,** p <0.01; B, Expression level of glycolytic-related enzyme protein after LY6K knockdown in A549 cells,** p <0.01,* p <0.05.

To observe the effect of LY6K on the growth of lung cancer in nude mice

As shown in Figure 6A, 1×10^7 cells were injected into the right upper armpit of nude mice as part of the pre-experiment. The tumor volume was then assessed every 7 days, and a growth curve was shown. Tumor growth was markedly reduced by LY6K knockout (p <0.01). Tumor size and weight of nude mice in the si-LY6K group were considerably lower than those in the control group after sampling (Fig. 6B-C, p <0.01), according to the findings. According to Western blot analysis, si-LY6K xenografts had substantially lower levels of the proteins GLUT1, HK2, PFKL, ALDOA, PGK-1, PKM2, and LDHA than the control group (Fig. 6D, p <0.05).

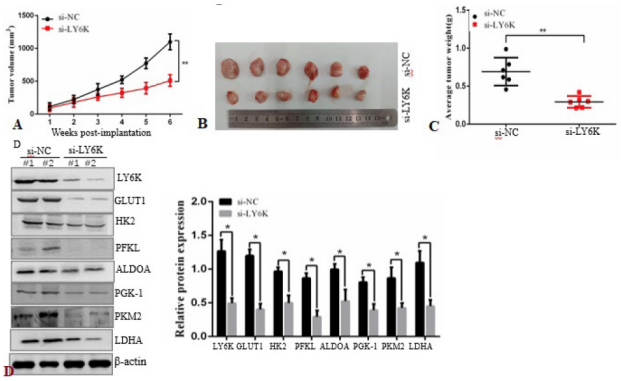

Fig. 6. The effect of LY6K knockdown in A549 cells on tumor formation was observed in subcutaneous tumor carrying experiment in nude mice. A, Tumor growth curves of si-NC group and si-LY6K group, ** p <0.01; B-C, Tumor weight in the si-NC group and the si-LY6K group, ** p <0.01; D, Expression of LY6K, GLUT1, HK2, PFKL, ALDOA, PGK-1, PKM2 and LDHA in subcutaneous tumor tissue of nude mice in si-NC group and si-LY6K group, * p <0.05.

LY6K promotes glycolysis of A549 cells

After transfecting si-NC and si-LY6K cells, the two

groups' glucose, lactic acid, and ATP concentrations were measured using a glucose, lactic acid, and ATP detection kit. According to the findings, the si-LY6K group's glucose absorption rate was much lower than that of the si-NC control group, and its levels of lactate generation and ATP concentration were both significantly lower. (p <0.05, Fig. 7) reveals that LY6K encouraged A549 cells' glycolysis.

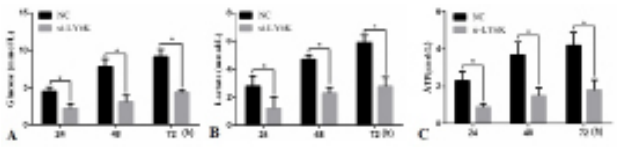

Fig. 7. Effect of LY6K knockdown on glycolysis of A549 cells. A, Glucose content in cell culture medium after LY6K knockdown by A549 cells, * p <0.05; B, Lactate content in cell culture medium after LY6K knockdown by A549 cells, * p <0.05; C, ATP concentration in cell culture medium after LY6K knockdown by A549 cells, * p <0.05.

DISCUSSION

A cancer/testicular antigen called LY6K is mostly or hardly expressed in normal tissue outside of the testicles but is strongly expressed in cancer tissue. The majority of research on LY6K in human disorders focuses mostly on how it affects cancer prognosis (Sastry *et al*., 2020; Guo *et al*., 2022). Uncertainty persists over the function of LY6K in the growth of cancer and its relationship to glucose metabolism.

The expression of LY6K was considerably elevated in lung cancer tissues and connected with a poor prognosis, which was consistent with other findings. In this investigation, we first evaluated the difference of LY6K mRNA expression in three databases (GEPIA2, TCGA, and Kaplan Meier plotter). Then, using a Western blot, LY6K protein expression was found in human normal BEAS-2b cells as well as the adenocarcinoma cells H1299, A549, and H358. The findings demonstrated that in A549 cells, LY6K expression was the most significant. A549 cells were selected as the primary study object as a result. We initially employed siRNA interference technology to knock off the expression of LY6K in A549 cells to evaluate the impact on cell proliferation before looking into the biological significance of LY6K in lung cancer cells. The results shown that LY6K knockdown might decrease the viability of lung cancer cells A549 and prevent tumor cell growth. Silencing LY6K caused G0/G1 phase arrest in A549 cells, according to flow cytometry. Cyclin and cyclin-dependent kinases are known to control the cell cycle in a precise manner (CDK). Our findings indicated that CyclinD1 and CDK4 expression levels in A549 cells were considerably reduced when LY6K was silenced, leading one to conclude that this resulted in G0/G1 phase arrest, which in turn inhibited the proliferation of A549 cells.

Aerobic glycolysis of tumors is considered to be a sign of rapid cell proliferation. Tumor cells need to consume more glucose than normal cells do in order to fulfill the demands of their fast multiplication. Tumor cells primarily depend on glycolysis for energy supply even in the presence of adequate oxygen. This metabolic feature is known as Warburg effect or aerobic glycolysis (Hanahan and Weinberg, 2011). Therefore, researchers speculated that regulating the expression of key glycolytic enzymes in tumor cells might be one of the feasible strategies for cancer treatment (Ghashghaeinia *et al*., 2019). At present, the main research involves glycolytic enzymes including hexokinase (HK), pyruvate kinase (PKM) and lactate dehydrogenation (LDH) (Sun *et al*., 2021; Shi *et al*., 2019; Zerhouni *et al*., 2021; Zhang *et al*., 2021). In this study, glycolytic related enzymes GLUT1, HK2, PFKL, ALDOA, PGK-1, PKM2 and LDHA were also downregulated after the knockdown of LY6K gene in A549 cells. *In vivo* tumor carrying experiments of nude mice, LY6K knockdown not only significantly inhibited the generation of transplanted tumors, but also significantly reduced the protein levels of GLUT1, HK2, PFKL, ALDOA, PGK-1, PKM2 and LDHA in xenograft tumor tissues, suggesting that the LY6K expression was related to glycolytic enzymes.

Changes in glucose consumption and lactate level can indirectly reflect the glycolysis level of cells. We found that after LY6K knockdown in A549 cells, the glucose uptake rate was greatly reduced, and the lactate production and ATP concentration were both significantly reduced. These findings suggested that LY6K knockdown might prevent A549 cells from undergoing glycolysis.

This study has several limitations. First, as opposed to using human samples, this research used an in vitro cell model. Second, since lung cancer A549 cells had the highest levels of LY6K expression, only these cells were examined in this investigation.

CONCLUSION

In conclusion, down-regulating LY6K can decrease the activity of key glycolytic enzymes in lung adenocarcinoma cells, obstruct the glycolytic process of tumor cells, and thereby inhibit the proliferation of lung adenocarcinoma cells.

ACKNOWLEDGEMENTS

Not applicable.

Funding

This research was funded by Medical Science Research Project of Hebei Provincial Health Commission 20221481 to Y.W.; Dr Fund of Hebei University of Chinese Medicine (BSZ2021022) to Y.W. and (BSZ2021007) to L.L.; Hebei Traditional Chinese Medicine Administration 2022368 to Y.W. and 2022080 to L.L.

Ethics approval and consent to participate

The experimental procedures and protocols were carried out in accordance with the ethical principles of animal experiments, and were approved by the Hebei University of Traditional Chinese Medicine.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Statement of conflict of interest

The authors have declared no conflict of interest.

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