

LncRNA NEAT1-associated aerobic glycolysis blunts tumor immunosurveillance by T cells in prostate cancer

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Long noncoding RNA (lncRNA) nuclear enriched abundant transcript 1 (NEAT1) is nuclear-located and transcribed from chromatin 11. To date, little is known about the cellular functions and regulatory mechanisms of NEAT1 in prostate cancer (PCa). In this study, whole-genome RNA sequencing data were downloaded from TCGA and GEO databases. Biological information was used to analyze the different expressions of NEAT1. In situ hybridization (ISH) was performed to detect the expression of NEAT1 in PCa and paracarcinoma clinical samples. Then, NEAT1 was knocked down in PC3 cells through lentiviral infection with a plasmid construct. Bioinformatics and integrative analytical approaches were utilized to identify the relationships of NEAT1 with specific cancer-related gene sets. Cell proliferation assay and colony formation assay were performed to evaluate the cell proliferative ability. Glycolysis stress test, metabolism assay, and infiltrating T-cell function analysis were implemented to assess the changes in metabolism and immune microenvironment of PCa. We found that the expression of NEAT1 was higher in PCa than in non-neoplastic tissues. The cell proliferative capability of PCa cells was significantly reduced in the NEAT1 knockdown group. PCR array and bioinformatics analysis revealed that the enrichment of acidic substance-related gene sets was associated with NEAT1 expression. NEAT1 depletion inhibited PCa cell aerobic glycolysis accompanied by the reduction of lactate levels in the medium. Further, we found that lactate dehydrogenase A (LDHA) expression was positively regulated by NEAT1. At last, co-culture systems indicated that NEAT1 or LDHA knockdown promoted the secretion of CD8⁺ T-lymphocyte factors, including TNF- α , IFN- γ , and Granzyme B, and enhanced the antitumor effects.

Key words: prostate cancer; NEAT1; aerobic glycolysis; tumor immunosurveillance

Prostate cancer (PCa) is a common malignant tumor of the male genitourinary system [1]. The incidence of PCa has increased, and PCa is one of the most commonly diagnosed malignancies in American and European men. Its mortality ranks third among male malignant tumors. It has become a major health concern in older male populations worldwide [2–4]. At present, radical prostatectomy and external radiation therapy are the main methods of treatment for localized PCa [5]. Hormonal therapy is also an important treatment method for patients with distant metastasis or recurrence [6]. Although this disease can be alleviated in most patients through the above treatments, the major challenge encountered in PCa therapy is that most patients will inevitably progress to castration-resistant prostate cancer (CRPC), which is considered the main cause of death among patients

with metastatic PCa [7]. The exact molecular mechanism underlying CRPC progression remains unclear. PCa research faces challenges in reaching its ultimate goal of CRPC treatment. First, we must find highly sensitive and specific biomarkers to distinguish CRPC from ADPC. Second, novel therapeutic strategies must be developed. Therefore, further studying the molecular mechanisms that underlie PCa progression is necessary to provide new approaches for the targeted therapy of PCa.

Long noncoding RNAs (lncRNAs) represent a large family of RNAs that are over 200 nucleotides in length and regulate various cellular events at the RNA level [8]. They are closely associated with the occurrence of cancers. A growing body of evidence has demonstrated that lncRNAs, such as ZEB1-AS1 and UCA1, play important regulatory roles in the

migration, proliferation, and invasion of cancer cells [9]. The nuclear enriched abundant transcript 1 (NEAT1) lncRNA is a nuclear-located ncRNA transcribed from chromatin 11 and an essential component of the structure of paraspeckles [10]. It mainly includes two isoforms, NEAT1_201 (3684 kb) and NEAT1_202 (22743 kb). Several independent studies have shown that NEAT1 is upregulated in different kinds of cancers, including gastric cancer, renal cancer, bladder cancer, non-small cell lung cancer, papillary thyroid cancer, endometrial carcinoma, hepatocellular carcinoma, gliomas, and ovarian cancer [11–16]. It plays key roles in a variety of cancer-related cellular activities, such as cell proliferation and apoptosis. In addition, it has been implicated in PCa. For example, NEAT1 has been reported to promote the invasive capability of PCa cells by enhancing the expression of CDC5L-AGRN [17]. Nevertheless, its exact role and molecular mechanism in metabolism and the immune microenvironment in the progression of PCa have not been elucidated.

In this study, we found that NEAT1 was elevated in PCa tissues relative to in paracancerous tissues and highly expressed in CRPC or Gleason ≥ 8 tissues relative to in ADPC or Gleason ≤ 7 tissues. In *in vitro* experiments, we found NEAT1 was elevated in PCa cells (PC3 and DU145) compared with prostate epithelial cells (HPEpiC), and in PC3 cells it was elevated more significantly. We selected PC3 and knocked down NEAT1 via the lentiviral infection of plasmid constructs to obtain cells with stable low NEAT1 expression. Then we performed cell proliferation experiments, cell plate cloning experiments, related metabolic and immune experiments. Compared with control cells, the NEAT1 knockdown group showed slower PCa cell proliferation, lower colony formation numbers, and significantly lower lactate expression and extracellular acidity rate (ECAR). After co-culturing with T cells, tumor cells in the knockdown group showed significantly increased levels of the cytokines TNF- α , INF- γ , and Granzyme B and inhibited proliferation compared with the control group.

Patients and methods

Data mining and bioinformatics analysis. Whole-genome RNA-seq data were downloaded from TCGA and GEO databases. Detailed information on the patients included in the datasets can be obtained from “The cBioPortal for Cancer Genomics”. Bioinformatics technology was used to analyze the differences between NEAT1 expression levels in PCa tissues and paracancerous tissues, in CRPC and ADPC tissues, and in Gleason ≥ 8 tissues and Gleason ≤ 7 tissues.

Cell culture and tissue collection. PCa cell lines were purchased from KeyGene Biotech (Shanghai, China). PC3 cells were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) supplemented with 10% fetal bovine serum (HyClone) and 1% penicillin-streptomycin. Cells were grown at 37°C in 5% CO₂. PCa tissues (n=85) and paracancerous tissues (n=54) were collected from the First Affili-

ated Hospital of the University of Science and Technology of China. The study protocol was approved by the ethics committee of the First Affiliated Hospital of the University of Science and Technology of China.

In situ hybridization (ISH) and immunohistochemical staining (IHC). Collected PCa and paracancerous tissues were subjected to in situ hybridization (ISH) and immunohistochemical staining (IHC). We performed ISH, firstly, antisense-locked nucleic acid-modified probes (RNA scope® Probe-Hs-NEAT1-short, ACD, NO.411531) were designed on the basis of the NEAT1 sequence, and the hybridized probes were incubated in 50% formamide hybridization buffer. Subsequently, samples were processed through probe hybridization. We performed IHC, primarily, selected appropriate LDHA antibodies (Abcam, NO.101562) according to the manufacturer's instructions. Then, PCa tissues and paracancerous tissues were stained with 3,3'-diaminobenzidine solution (DAB). Three independent ISH and IHC experiments were repeated, and stained tissues were imaged with BX-60 and BX-16 microscopes (Olympus, Japan).

Establishment of stable cells. The lentiviral plasmids p-random, p-shRNA1, and p-shLDHA were co-transfected with plasmids encoding polymerase, group antigen, vesicular stomatitis virus G protein, and envelope protein into HEK293T cells by using Lipofectamine 2000 (Invitrogen). Viral supernatant was collected at 48h post-transfection and added to PC3 cells in the presence of 8 μ g/ml polybrene (Sigma-Aldrich). Transduced PC3 cells were selected with 1 μ g/ml puromycin. shRNAs targeting NEAT1 and LDHA were commercially purchased (Shanghai GenePharma Co., Ltd.) and had the following sequences: sh-NEAT1 Forward: TGGTAATGGTGGAGGAAGA; sh-NEAT1 reverse: TCTTCCCTCCACCATTACCA; sh-LDHA forward: AAGACATCATCCTTTATTCCG; and sh-LDHA reverse: CGGAATAAAGGATGATGTCTT.

RNA extraction and real-time PCR. Total RNA was isolated by using TRIzol reagent (Ambion, Cat. No. 15596) in accordance with the manufacturer's instructions. After RNA extraction, RNA samples were reverse-transcribed by using a High-Capacity cDNA Reverse Transcription Kit (Promega, Cat. No. A5001). The real-time qPCR experiment was performed on a Bio-Rad CFX96 qPCR system in accordance with the manufacturers' instructions (Vazyme, Cat. No. Q111-03). The primers used to detect the expression of NEAT1 and GAPDH (internal control) were as follows: NEAT1 forward: CAATTACTGTCGTTGGGATTTAG-AGTG; reverse: TTCTTACCATACAGACAACATACCAG; GAPDH forward: CATGTTTCGTCATGGGTGTGAACCA; and reverse: ATGGCATGGACTGTGGTCATGAGT; LDHA forward: ACATCCTGGGCTATTGGACT; reverse: TTCTTCAAACGGCCCTCTTC. The relative fold changes of candidate genes were analyzed by using the 2^{- $\Delta\Delta$ CT} method.

Cell proliferation assay. The cell proliferation assay was performed in accordance with the manufacturer's instruc-

tions. Briefly, PC3 cells were plated in 96-well plates at the density of 5000 cells per well. Cells were incubated in RPMI-1640 media with 10% FBS for 24, 48, 72, and 96 h. The media were aspirated from each well and replaced with 100 μ l of a mixture containing 90 μ l of medium and 10 μ l of solution reagent. The plates were incubated for 2 h at 37°C in a cell incubator. Absorbance was measured at 450 nm by using a microplate reader.

Colony formation assay. Five hundred cells of the sh-NEAT1 and negative control groups were plated on six-well plates with 2 ml of RPMI-1640 medium per well. The medium was replaced once every 2 days. After 1 week, the medium was aspirated, and the cells were washed twice with 1 ml of PBS. Then, 2 ml of 4% paraformaldehyde was added to each well for 15 min. Each well was stained with 2 ml of crystal violet for 10 min and washed thrice with 2 ml of distilled water. The plates were inverted for drying. Images were acquired, and clone numbers were counted under a microscope.

NuRNA human central metabolism PCR array and clustering heatmap construction. PC3 cells from the sh-NEAT1 and control groups were subjected to NuRNA Human Central Metabolism PCR Array by the Shanghai Kangcheng Biological Company (China) (<http://www.aksomics.com/services/transcriptomics/arraystar-mrna-pcr-array.html>). Briefly, PC3 cells were used for mRNA microarray assays. Total RNA was extracted and evaluated for quality through agarose gel electrophoresis. Then, cDNA synthesis and RT-qPCR were performed. Data were analyzed, and several differential genes from the test results were selected. The Heatmapper online mapping tool (<http://www.heatmapper.ca>) was used to prepare clustering heat maps.

Metabolism assay and glycolysis stress test. A total of 1.0×10^7 PC3 cells from the sh-NEAT1 and negative control groups were plated on six-well plates with 2 ml of RPMI-1640 medium per well. To determine the levels of glucose and lactate in the cells, when the cell density reached 90%, the medium was removed and assayed for glucose and lactate levels by using a glucose assay kit and lactate assay kit (BioVision, USA) in accordance with the manufacturer's instructions. Cells were collected and the amount of protein was detected. The remaining glucose and lactate productions were calculated based on the standard curve and normalized to the cell number.

As for the glycolysis stress test, the Seahorse XF96 analyzer glycolysis method was used to measure ECAR to calculate net proton production in the extracellular medium. The degree of the acidification of the medium surrounding cells during glycolysis was directly measured by using the XF analyzer and reported as ECAR.

Western blot analysis. PC3 cells were lysed in RIPA buffer supplemented with 1% proteinase inhibitors (PIC) and 1 mM PMSF (Beyotime, Hangzhou, China) for total protein preparation. Primary antibodies used were LDHA-labeled

anti-rabbit (1:5000, Proteintech, NO. 21799-1-AP) and β -actin-labeled anti-mouse (1:1000, Abcam, NO. 8226), and secondary antibody used goat anti-rabbit IgG-HRP (1:5000, Proteintech, NO. SA00001-2), goat anti-mouse IgG-H+L (1:5000, Proteintech, NO. SA00001-1).

T-cell isolation and co-culture study. Human peripheral blood mononuclear cells (PBMCs) were isolated from adult blood buffy coat samples from healthy donors (obtained from the First Affiliated Hospital of University of Science and Technology of China) via Ficoll (Ficoll-Paque PLUS-17144002-1, GE Healthcare) density gradient centrifugation in accordance with the manufacturer's protocol. Purified NA/LE Mouse Anti-Human CD3 (BD Pharmingen™, NO. 555329) was added to a 96-well plate and incubated overnight at 4°C. PBMCs were plated in 96-well plates at a density of 2×10^5 cells and followed by adding purified NA/LE Mouse Anti-Human CD28 (BD Pharmingen™, NO.555725) and keeping in a humid incubator at 37°C and 5% CO₂ for 2 days. Then, PCa cell culture medium was added at the PCa cell density of 80–90% and the plates were incubated at 37°C in 5% CO₂ for 3 days. Later, a cocktail (eBioscience™ Cell Stimulation Cocktail, NO.00-4975) was added to stimulate cytokine generation. After 6 h, the cells were collected, resuspended in PBS, and labeled with antibodies (Hu CD8 APC RPA-T8-561952, Hu CD4 FITC RPA-T4, BD Bioscience) to screen CD8⁺ and CD4⁺ cell population, away from light at 4°C for 20 min. After washing with PBS, the cell membranes were ruptured with 100 μ l BD Cytotfix/Cytoperm (BD, NO.554714) and kept away from light for 20 min. Antibodies (Humn IFN-Gma PE-CY7S B3-561036, Hu TNF BV421 Mab11-566275, and Hu Granzyme B PE GB11-561142, BD Bioscience) were used for the detection of intracellular cytokines via flow cytometry.

Statistical analysis. Each value was obtained from at least three independent experiments and presented as mean \pm SD. The significance of differences among the means was calculated by using t-tests, chi-square test, for the two-group comparisons, and the expressive relation between LDHA and NEAT1 was assessed by Pearson's correlations analysis with Statistical Package of the Social Sciences software version 22.0 (Inc. Chicago, IL, USA). A two-sided p-value of <0.05 was considered statistically significant. In all Figures, *, ** and *** represent $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

Results

Expression of NEAT1 was higher in prostate tissues than non-cancerous tissues. By using TCGA data and GEO data, we initially analyzed NEAT1 expression levels in paracancerous and PCa tissues and further compared the difference in expression at different stages, and in PCa tissues in different pathological stages. We found that NEAT1 expression in PCa tissues was elevated relative to that in paracancerous tissues (Figure 1A). Furthermore, NEAT1 expression was higher in Gleason ≥ 8 PCa tissues

than in Gleason ≤ 7 tissues (Figure 1B). In addition, NEAT1 expression in CRPC significantly increased compared with that in ADPC (Figure 1C). Finally, we collected 139 clinical samples, including 85 cases of PCa tissues and 54 cases of paracancerous tissues, and detected NEAT1 expression through in situ hybridization (ISH). The results showed that the NEAT1 staining intensity of PCa tissues was higher than that of paracancerous tissues (as shown in the representative samples in Figures 1D, 1E), indicating that NEAT1 expression was significantly higher in PCa tissues relative to that in paracancerous tissues (Figure 1F).

In vitro functional experiments on the biological function of NEAT1 in PCa cells. We measured NEAT1 expression levels in human prostate epithelial cells (HPEpiC) and PCa cell lines (PC3 and DU145) and found it was elevated in PC3 and DU145 cells compared with HPEpiC cells, and in PC3 cells it was elevated more significantly (Figure 2A). Then, we knocked down NEAT1 via the lentiviral infection of plasmid constructs into PC3 cells to obtain cells with stable low NEAT1 expression to further study the biological function of NEAT1 (Figure 2B). We examined the impact of NEAT1 expression on cell proliferation in PC3 cell lines. The results showed that the cell proliferative capability of original PC3 lines was significantly enhanced compared with that of the knockdown group (Figure 2C). We also performed colony formation assays to further explore the effect of NEAT1 on PC3 cells and found that the colony number formed in the

knockdown group was significantly lower than that in the control group (Figures 2D, 2E).

NEAT1 expression influenced glucose metabolism and is positively correlated with LDHA in PCa. NuRNA Human Central Metabolism PCR Array was performed in the control group and the NEAT1 knockdown group. A series of genes related to glucose metabolism were dysregulated. (Figure 3A). Bioinformatics analysis revealed that the enrichment of acidic substance-related gene sets was associated with NEAT1 expression (Figure 3B). Firstly, we tested extracellular acidity rate (ECAR) in PC3 cell, which has been widely used in our previous studies, and found that the sh-NEAT1 group showed reductions in basal glycolysis capability, maximum glycolysis capability, and glycolysis reserve capability compared with the control group (Figures 3C, 3D). Second, we measured glucose and lactate levels in the medium to clarify the effect of NEAT1 expression on the function of glucose metabolism. We found that the remaining glucose was significantly higher in PC3 cells of the sh-NEAT1 group, but lactate production in PC3 cells of the sh-NEAT1 group was lower than that in the control group (Figures 3E, 3F). To determine if NEAT1 regulates LDHA expression, we detected LDHA expression in the NC and sh-NEAT1 group in PC3 cells and found that the LDHA expression in the sh-NEAT1 group was significantly reduced compared to the NC group (Figure 3G). In situ hybridization and immunohistochemistry were used to detect the expres-

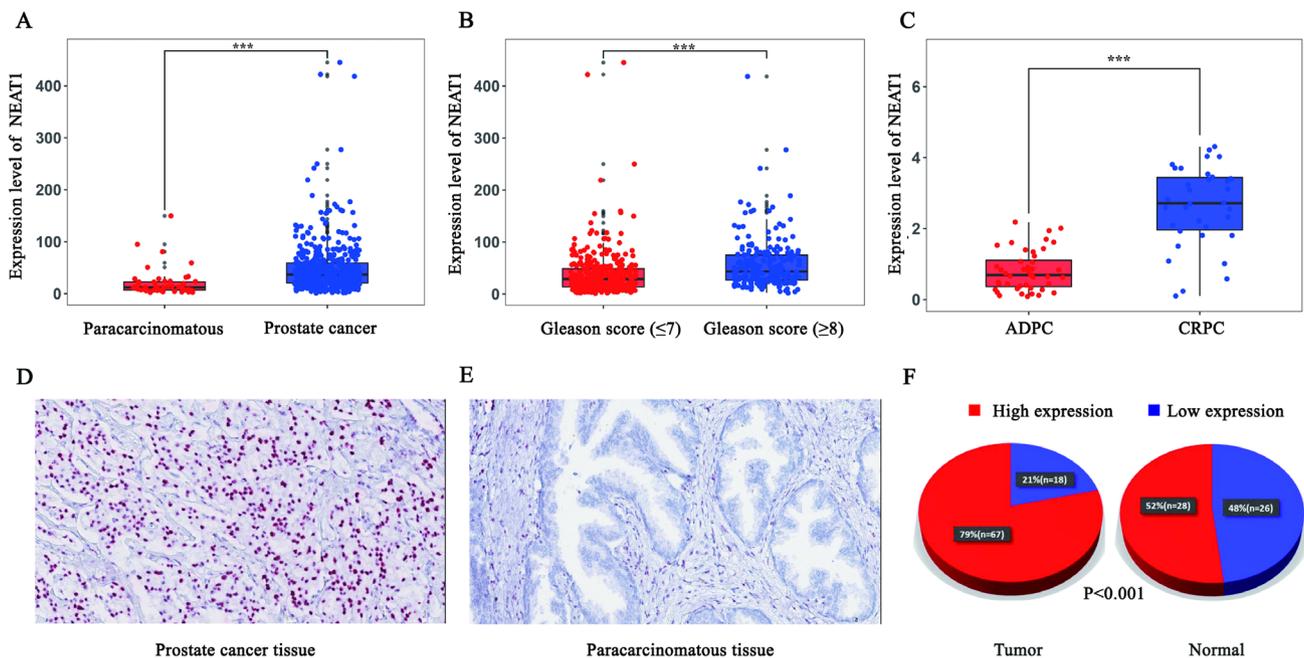


Figure 1. The expression of NEAT1 in PCa tissues and non-cancerous tissues. A) NEAT1 expression was elevated in PCa tissues compared with paracarcinoma tissues. B) NEAT1 expression was higher in Gleason score ≥ 8 prostate cancer tissues compared with Gleason score ≤ 7 ones. C) NEAT1 expression significantly increased in CRPC compared with ADPC. D, E) ISH were performed on prostate cancer tissues and non-carcinoma tissues, NEAT1 was strongly expressed in most of the prostate cancer tissues with brown staining (shown at right) but absent in most of the non-carcinoma tissues with blue staining (shown at left). F) The difference was statistically significant.

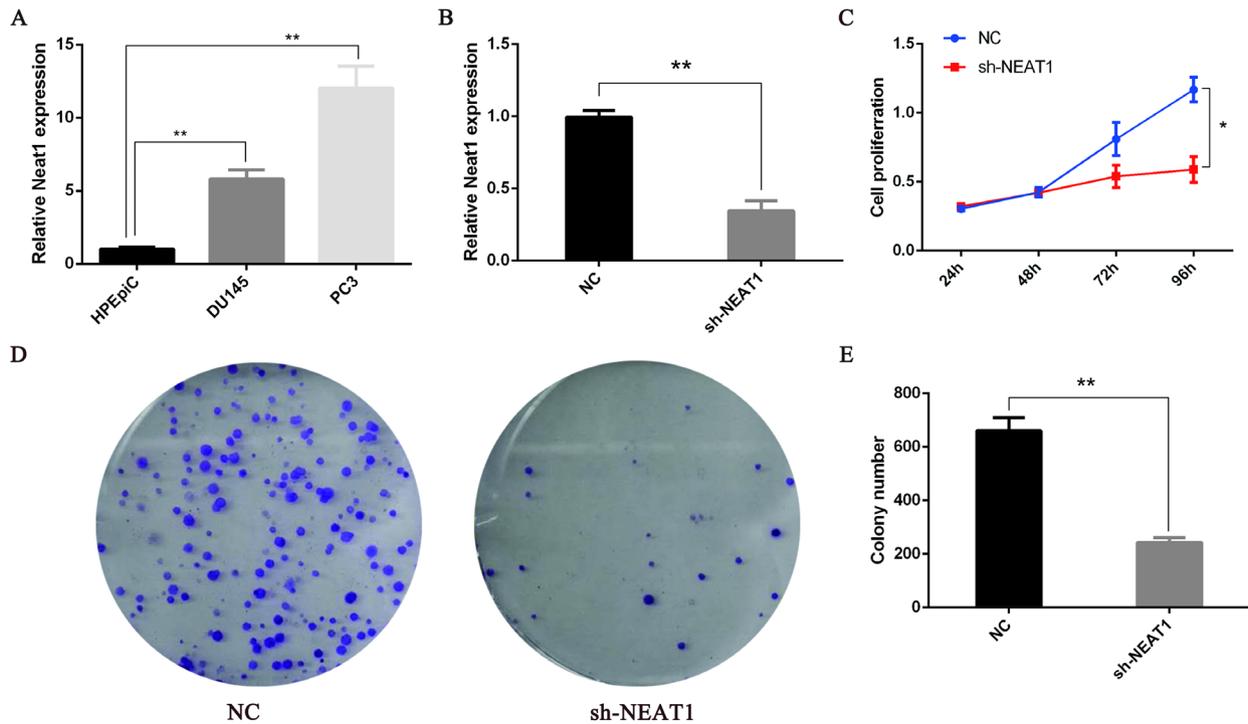


Figure 2. The function of NEAT1 in PC3 cell line. A) The expression of NEAT1 was elevated in PC3 and DU145 cells compared with HPEpiC cells, and in PC3 cells it was elevated more significantly. B) The expression of NEAT1 was knocked down via the lentiviral infection of plasmid constructs into PC3 cells to obtain cells with stable low NEAT1 expression and measured by RT-qPCR. C) CCK-8 assay revealed that the decreased NEAT1 expression significantly decreased the proliferative index of PC3 cells. D, E) The results of colony formation assay revealed that knockdown NEAT1 results in a reduced number of plate clones in PC3 cells.

sion of NEAT1 and metabolism-related protein. Pearson correlations showed a significant positive correlation between NEAT1 and LDHA levels ($p < 0.001$, Figures 3H, 3I).

NEAT1 knockdown enhanced the antitumor effects of T lymphocytes. Some articles have reported that an acidic cell environment inhibits the immune surveillance function of T cells and is associated with poor outcomes in patients with tumors [18]. We developed an *in vitro* co-culture model and used PC3 cell lines to verify functionally the effect of an acidic microenvironment on the antitumor effect of T lymphocytes. We co-cultured the medium of sh-NEAT1 group cells with T lymphocytes, separated the CD8⁺ lymphocyte subsets by flow cytometry, and then detected the intracellular expression levels of the IFN- γ , TNF- α , and Granzyme B factors secreted by CD8⁺ cells. And we found that cytokine expression in the sh-NEAT1 group was higher than that in the control group (Figures 4A, 4B). In addition, the glycolysis stress assay as described above revealed that the extracellular acidity rate of the sh-NEAT1 group decreased compared with the control group. It indicated that an acidic microenvironment was not conducive to CD8⁺ lymphocytes to secrete cytokine. We consider the increased levels of cytokines in the sh-NEAT1 group may be related to the reduction of LDHA. For further verification, we knocked down the LDHA in PC3

cells (Figure 4C). Then, we co-cultured the medium of the sh-LDHA group and the NC group cells with T lymphocytes. The result indicated that the cytokines expression in the sh-LDHA group was higher than that in the NC group (Figures 4D, 4E), which was similar to the sh-NEAT1 group.

Discussion

In our study, NEAT1 expression in PCa tissues significantly increased compared with that in paracancerous tissues. Similarly, NEAT1 expression increased in CRPC tissues relative to that in ADPC tissues. NEAT1 expression was also significantly elevated in Gleason ≥ 8 PCa tissues compared with that in Gleason ≤ 7 PCa tissues. NEAT1 knockdown inhibited cell proliferation capability and cloning capacity *in vitro*. Additionally, metabolism chip analysis revealed that some glucose metabolism-related genes were expressed at low levels in the sh-NEAT1 group. And through bioinformatics analysis, we found that the enrichment of acidic substance-related gene sets was associated with NEAT1 expression. Then, we performed a glycolysis stress test and found aerobic glycolysis was inhibited and ECAR decreased in the sh-NEAT1 group. Further, we found that LDHA expression was positively regulated by NEAT1. Lastly, we

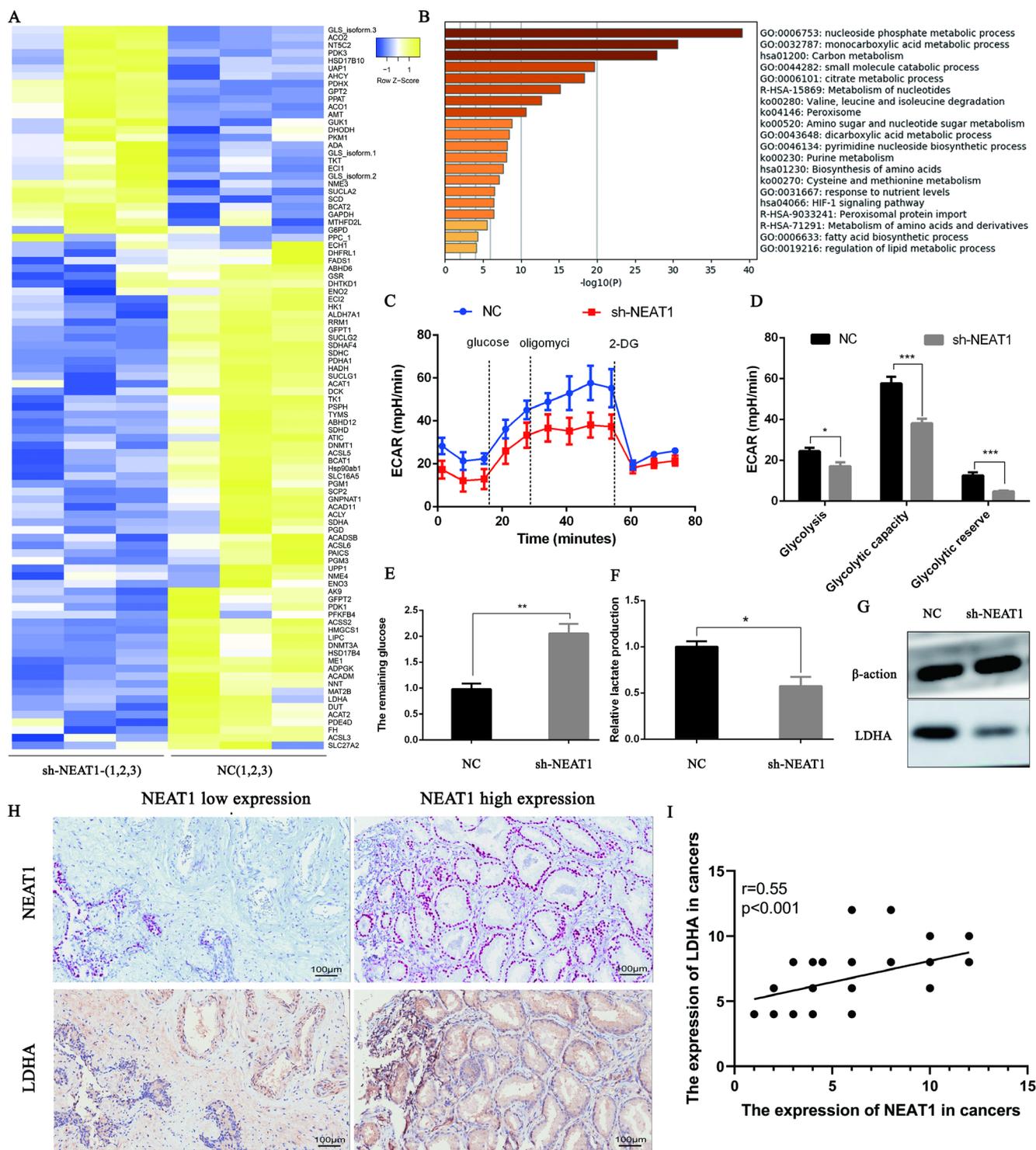


Figure 3. NEAT1 expression influenced glycolysis and is positively correlated with LDHA in PCa. A) We implemented NuRNA Human Central Metabolism PCR Array and discovered that the expression of a series of genes related to glucose metabolism was dysregulated. B) Bioinformatics analysis revealed that the enrichment of acidic substance-related gene sets was associated with NEAT1 expression. C, D) Knockdown of NEAT1 weakened the glycolytic capacities of PCa cells compared with negative control. E) The glucose absorption capacity was significantly higher in the sh-NEAT1 group than in the NC group. F) Extracellular lactate production levels were significantly lower in the sh-NEAT1 group than in the NC group. G) The expression of LDHA in the sh-NEAT1 group was lower than that in the NC group in PCa cells. H) In situ hybridization and immunohistochemistry were used to detect the expression of NEAT1 and LDHA. I) Pearson correlations showed a significant positive correlation between NEAT1 and LDHA levels.

found NEAT1 or LDHA knockdown promoted the secretion of CD8⁺ T lymphocyte factors, including TNF- α , IFN- γ , and Granzyme B, and enhanced antitumor effects. Our results showed that NEAT1 played an important role in suppressing the immune surveillance of T cells at least partially through regulating LDHA-mediated aerobic glycolysis in PCa.

An increasing number of studies have confirmed that metabolic disorders are closely related to the occurrence of cancer. Some reports have suggested that diets high in polyunsaturated fatty acids are negatively associated with cancer development and that the end product of arachidonic acid metabolism (PEG2) induces tumor occurrence by inhibiting immunity [19–20]. In addition, growing evidence demonstrates that amino acids are essential nutrients for cancer growth and are used by tumors in various biosynthetic pathways and as a source of energy [21]. One of the most distinguishing characteristics between normal and tumorigenic cells is altered glucose metabolism, which is considered to be a new feature of tumorigenesis [22]. In the presence of oxygen, normal cells produce a molecule of glucose through the Krebs cycle and 38 ATP molecules through oxidative

phosphorylation; in the absence of oxygen, cells under anaerobic glycolysis produce two molecules of ATP [23]. However, tumor cells exhibit active glycolysis and metabolize additional glucose into lactate in aerobic and hypoxic environments. This specific metabolic process is called the Warburg effect [24]. It may be related to incomplete mitochondrial function in tumor cells and may promote cancer cell invasion capacity and apoptosis resistance [25, 26]. In this research, we examined lactate expression in media from PC3 cell lines in the control and sh-NEAT1 groups and found that lactate expression in the sh-NEAT1 group was lower than that in the control group. Furthermore, the result of the glycolysis stress test suggested that ECAR in the knockdown group was lower than that in the control group. This result demonstrated that knocking down NEAT1 changed the glycolysis function of PCa cells and promoted the occurrence of aerobic glycolysis.

Tumor-infiltrating immune cells play a key role in controlling tumor development [27]. T lymphocytes are the most abundant and best-characterized immune cells in the tumor microenvironment; they mediate cellular immunity by inhibiting tumor initiation and progression and have thus emerged

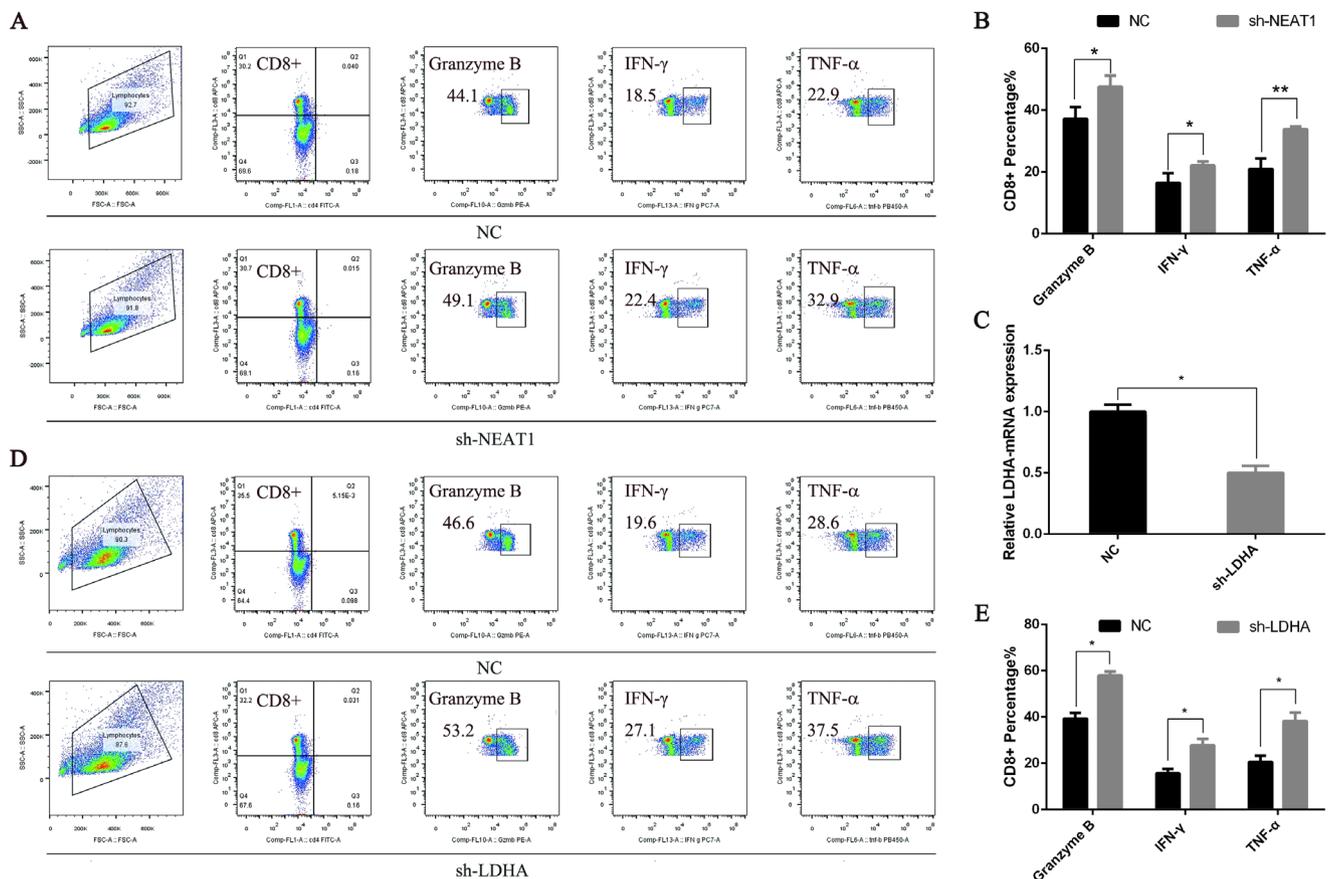


Figure 4. sh-NEAT1 increases anti-tumor effects of CD8⁺ T lymphocytes. A, B) The expression of Granzyme B, IFN- γ , and TNF- α of CD8⁺ cells was increased when co-cultured in the sh-NEAT1 PC3 cell culture medium. C) PC3 cells had stable low LDHA expression via the lentiviral infection and measured by RT-qPCR. The LDHA-mRNA expression of the sh-LDHA group was decreased compared with the NC group. D, E) The expression of Granzyme B, IFN- γ , and TNF- α of CD8⁺ cells was raised after co-culturing with the sh-LDHA group PC3 cell culture medium.

as a novel strategy for treating various cancers [28]. However, in an acidified tumor microenvironment, T lymphocytes decrease the production of cytokines, particularly IFN- γ , TNF- α , and Granzyme B, thus weakening antitumor effects [29]. Mechanistically, lactate uptake by CD8⁺ T cells promotes intracellular acidification, which disturbs the transcription of cytokines by inhibiting transcription factors, destroying tumor immunosurveillance, and further enhancing tumor growth [30]. In this research, we co-cultured T lymphocytes in PCa cell medium and detected cytokine levels in the culture media. We found that IFN- γ , TNF- α , and Granzyme B levels in the knockdown group were significantly higher than those in the control group, and cells proliferated more slowly in the knockdown group in the control group. These results may be related to the low lactate levels in the knockdown group. Taken together, these results indicated that an acidic microenvironment regulated PCa immunity in multiple ways, and its continued exploitation would significantly advance immunotherapeutic approaches for PCa.

Above all, we found acidic microenvironment inhibits the proliferation of prostate cancer cells. It may be related to the acidic microenvironment interfering with the function of T lymphocytes and inhibiting the killing effect of T lymphocytes on tumor cells. Furthermore, we discovered that NEAT1 could regulate glucose metabolism in PCa, knockdown it can decrease extracellular acidity rate, and inhibited lymphokine secretion by T lymphocytes when cocultured with PCa cell medium. These results established a foundation for further research on the antitumor effects of T lymphocytes. Next, we aim to reveal the specific mechanism, provide novel targets, and contribute to optimizing molecular targeted treatment strategies for PCa.

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References

- [1] TAO T, CHEN M, JIANG R, GUAN H, HUANG Y et al. Involvement of EZH2 in aerobic glycolysis of prostate cancer through miR-181b/HK2 axis. *Oncol Rep* 2017; 37: 1430–1436. <https://doi.org/10.3892/or.2017.5430>
- [2] SIEGEL RL, MILLER KD, FUCHS HE, JEMAL A. Cancer Statistics, 2021. *CA Cancer J Clin* 2021; 71: 7–33. <https://doi.org/10.3322/caac.21654>
- [3] XIONG W, HUANG C, DENG H, JIAN C, ZEN C et al. Oncogenic non-coding RNA NEAT1 promotes the prostate cancer cell growth through the SRC3/IGF1R/AKT pathway. *Int J Biochem Cell Biol* 2018; 94: 125–132. <https://doi.org/10.1016/j.biocel.2017.12.005>
- [4] SCHATTEN H. Brief Overview of Prostate Cancer Statistics, Grading, Diagnosis and Treatment Strategies. *Adv Exp Med Biol* 2018; 1095: 1–14. https://doi.org/10.1007/978-3-319-95693-0_1
- [5] WILT TJ, ULLMAN KE, LINSKENS EJ, MACDONALD R, BRASURE M et al. Therapies for Clinically Localized Prostate Cancer: A Comparative Effectiveness Review. *J Urol* 2021; 205: 967–976. <https://doi.org/10.1097/JU.0000000000001578>
- [6] TUCCI M, LEONE G, BUTTIGLIERO C, ZICHI C, STEFANO RFD et al. Hormonal treatment and quality of life of prostate cancer patients: new evidence. *Minerva Urol Nefrol* 2018; 70: 144–151. <https://doi.org/10.23736/S0393-2249.17.03066-1>
- [7] LING Z, WANG X, TAO T, ZHANG L, GUAN H et al. Involvement of aberrantly activated HOTAIR/EZH2/miR-193a feedback loop in progression of prostate cancer. *J Exp Clin Cancer Res* 2017; 36: 159. <https://doi.org/10.1186/s13046-017-0629-7>
- [8] MORETTO F, WOOD NE, KELLY G, DONCIC A, VAN WERVEN FJ et al. A regulatory circuit of two lncRNAs and a master regulator directs cell fate in yeast. *Nat Commun* 2018; 9: 780. <https://doi.org/10.1038/s41467-018-03213-z>
- [9] ZHAO X, WANG D, DING Y, ZHOU J, LIU G et al. lncRNA ZEB1-AS1 promotes migration and metastasis of bladder cancer cells by post-transcriptional activation of ZEB1. *Int J Mol Med* 2019; 44: 196–206. <https://doi.org/10.3892/ijmm.2019.4187>
- [10] WEST JA, DAVIS CB, SUNWOO H, SIMON MD, SADDREYEV RI et al. The long noncoding RNAs NEAT1 and MALAT1 bind active chromatin sites. *Mol Cell* 2014; 55: 791–802. <https://doi.org/10.3892/ijmm.2019.4187>
- [11] FU JW, KONG Y, SUN X. Long noncoding RNA NEAT1 is an unfavorable prognostic factor and regulates migration and invasion in gastric cancer. *J Cancer Res Clin Oncol* 2016; 142: 1571–1579. <https://doi.org/10.1007/s00432-016-2152-1>
- [12] NING L, LI Z, WEI D, CHEN H, YANG C. LncRNA, NEAT1 is a prognosis biomarker and regulates cancer progression via epithelial-mesenchymal transition in clear cell renal cell carcinoma. *Cancer Biomark* 2017; 19: 75–83. <https://doi.org/10.3233/CBM-160376>
- [13] WANG Y, DU L, YANG X, LI J, LI P et al. A nomogram combining long non-coding RNA expression profiles and clinical factors predicts survival in patients with bladder cancer. *Aging (Albany NY)* 2020; 12: 2857–2879. <https://doi.org/10.18632/aging.102782>
- [14] WANG S, CUI Z, LI H, LI J, LV X et al. LncRNA NEAT1 polymorphisms and lung cancer susceptibility in a Chinese Northeast Han Population: A case-control study. *Pathol Res Pract* 2019; 215: 152723. <https://doi.org/10.1016/j.prp.2019.152723>
- [15] ZHANG H, CAI Y, ZHENG L, ZHANG Z, LIN X et al. Long noncoding RNA NEAT1 regulate papillary thyroid cancer progression by modulating miR-129-5p/CLK7 expression. *J Cell Physiol* 2018; 233: 6638–6648. <https://doi.org/10.1002/jcp.26425>

- [16] SHAKER OG, ABDELWAHED MY, AHMED NA, HASSAN EA, AHMED TI et al. Evaluation of serum long non-coding RNA NEAT and MiR-129-5p in hepatocellular carcinoma. *IUBMB Life* 2019; 71: 1571–1578. <https://doi.org/10.1002/iub.2096>
- [17] LI X, WANG X, SONG W, XU H, HUANG R et al. Oncogenic Properties of NEAT1 in Prostate Cancer Cells Depend on the CDC5L-AGRN Transcriptional Regulation Circuit. *Cancer Res* 2018; 78: 4138–4149. <https://doi.org/10.1158/0008-5472.CAN-18-0688>
- [18] ZHANG W, WANG G, XU ZG, TU H, HU F et al. Lactate Is a Natural Suppressor of RLR Signaling by Targeting MAVS. *Cell* 2019; 178: 176–189.e115. <https://doi.org/10.1016/j.cell.2019.05.003>
- [19] MIZUNO R, KAWADA K, SAKAI Y. Prostaglandin E2/EP Signaling in the Tumor Microenvironment of Colorectal Cancer. *Int J Mol Sci* 2019; 20: 6254. <https://doi.org/10.3390/ijms20246254>
- [20] LI Z, WEI D, YANG C, SUN H, LU T et al. Overexpression of long noncoding RNA, NEAT1 promotes cell proliferation, invasion and migration in endometrial endometrioid adenocarcinoma. *Biomed Pharmacother*. 2016; 84: 244–251. <https://doi.org/10.1016/j.biopha.2016.09.008>
- [21] ANANIEVA EA, WILKINSON AC. Branched-chain amino acid metabolism in cancer. *Curr Opin Clin Nutr Metab Care* 2018; 21: 64–70. <https://doi.org/10.1097/MCO.0000000000000430>
- [22] CHANG CH, QIU J, O'SULLIVAN D, BUCK MD, NOGUCHI T et al. Metabolic Competition in the Tumor Microenvironment Is a Driver of Cancer Progression. *Cell* 2015; 162: 1229–1241. <https://doi.org/10.1016/j.cell.2015.08.016>
- [23] HUI S, GHERGUROVICH JM, MORSCHER RJ, JANG C, TENG X et al. Glucose feeds the TCA cycle via circulating lactate. *Nature* 2017; 551: 115–118. <https://doi.org/10.1038/nature24057>
- [24] SUN L, SUO C, LI ST, ZHANG H, GAO P. Metabolic reprogramming for cancer cells and their microenvironment: Beyond the Warburg Effect. *Biochim Biophys Acta Rev Cancer* 2018; 1870: 51–66. <https://doi.org/10.1016/j.bbcan.2018.06.005>
- [25] PORPORATO PE, FILIGHEDDU N, PEDRO JMB, KROEMER G, GALLUZZI L. Mitochondrial metabolism and cancer. *Cell Res* 2018; 28: 265–280. <https://doi.org/10.1038/cr.2017.155>
- [26] FIELD CS, BAIXAULI F, KYLE RL, PULESTON DJ, CAMERON AM et al. Mitochondrial Integrity Regulated by Lipid Metabolism Is a Cell-Intrinsic Checkpoint for Treg Suppressive Function. *Cell Metab* 2020; 31: 422–437.e425. <https://doi.org/10.1016/j.cmet.2019.11.021>
- [27] FARHOOD B, NAJAFI M, MORTEZAEE K. CD8(+) cytotoxic T lymphocytes in cancer immunotherapy: A review. *J Cell Physiol* 2019; 234: 8509–8521. <https://doi.org/10.1002/jcp.27782>
- [28] HO PC, BIHUNIAK JD, MACINTYRE AN, STARON M, LIU X et al. Phosphoenolpyruvate Is a Metabolic Checkpoint of Anti-tumor T Cell Responses. *Cell* 2015; 162: 1217–1228. <https://doi.org/10.1016/j.cell.2015.08.012>
- [29] SHI L, FENG M, DU S, WEI X, SONG H et al. Adenosine Generated by Regulatory T Cells Induces CD8(+) T Cell Exhaustion in Gastric Cancer through A2aR Pathway. *BioMed Res Int* 2019; 2019: 4093214. <https://doi.org/10.1155/2019/4093214>
- [30] BRAND A, SINGER K, KOEHL GE, KOLITZUS M, SCHOENHAMMER G et al. LDHA-Associated Lactic Acid Production Blunts Tumor Immunosurveillance by T and NK Cells. *Cell Metab* 2016; 24: 657–671. <https://doi.org/10.1016/j.cmet.2016.08.011>