Study on the Role of TIMP1 and DPP4 in the Development of Lactic Acid Metabolism and PTC

Shiji Mu , Xue Jin , Fada Xia , Xiwu Ouyang , Guode Fu , Ruotong Gui , Haihong Wang , Ning Bai *

Posted Date: 13 March 2026

doi: 10.20944/preprints202603.1110.v1

Keywords: papillary thyroid carcinoma; cell proliferation; lactate metabolism; immune infiltration; drug sensitivity

 doi:10.20944/preprints202603.1110.v1

Article

Study on the Role of TIMP1 and DPP4 in the Development of Lactic Acid Metabolism and PTC

Shiji Mu [1], Xue Jin [2], Fada Xia [3], Xiwu Ouyang [3], Guode Fu [1], Ruotong Gui [1], Haihong Wang [1] and Ning Bai [3,4,*]

[1] The Second Affiliated Hospital of Xinjiang Medical University, Urumqi, Xinjiang

[2] Changji People's Hospital, Urumqi, Xinjiang

[3] Department of General Surgery, Xiangya Hospital, Central South University, Changsha, Hunan Province

[4] The Second Affiliated Hospital of Xinjiang Medical University, Department of Oncology, Urumqi, Xinjiang

* Correspondence: baining@csu.edu.cn; Tel.: +8613975804018

Simple Summary

The genes related to lactate metabolism play a significant role in the occurrence and development of various tumors. The mechanism of their action in papillary thyroid carcinoma (PTC) still needs further research and exploration. In this study, key genes related to lactate metabolism that function in PTC were selected from the known genes related to lactate metabolism. Bioinformatics analysis, experimental verification, and exploration of the mechanism of these genes in PTC were conducted, and their role in promoting lactate metabolism in PTC cells was investigated. The results showed that compared with normal PTC cells, TIMP1 and DPP4 were highly expressed in thyroid papillary carcinoma. Silencing the expression of TIMP1 and DPP4 with siRNA could reduce the invasion and proliferation ability of PTC cells. Compared with normal thyroid cells, the content of lactate and LDHA in PTC cells was higher. Knocking down the expression of TIMP1 and DPP4 reduced the lactate production ability of PTC cells, and TIMP1 and DPP4 promoted the accumulation of lactate in PTC cells.

Abstract

Objectives: The mechanism of action of genes related to lactate metabolism in papillary thyroid carcinoma (PTC) is still unclear. In this study, key genes that play a role in PTC were selected from the known genes related to lactate metabolism, and their roles in promoting lactate metabolism in PTC cells were investigated.**Methods**: Through bioinformatics analysis and cell experiments, the roles of the relevant genes in lactate metabolism and their roles in the occurrence and development of PTC were verified.**Results**: Through bioinformatics analysis, 12 candidate genes were obtained. Through qRT-PCR experiments, it was confirmed that the expressions of TIMP1 and DPP4 were higher in thyroid papillary carcinoma than in normal PTC cells. By inhibiting the expression of TIMP1 and DPP4 using siRNA, the invasion and proliferation abilities of PTC could be reduced. Compared with normal thyroid cells, the contents of lactic acid and LDHA in PTC cells were higher. Knocking down the expression of TIMP1 and DPP4 would reduce the lactate production ability of PTC cells, and TIMP1 and DPP4 promoted the accumulation of lactate in PTC cells.

Keywords: papillary thyroid carcinoma; cell proliferation; lactate metabolism; immune infiltration; drug sensitivity

1. Introduction

Thyroid cancer is the most common endocrine malignancy worldwide [1], accounting for 3.4% of all diagnosed tumors [2]. Papillary thyroid carcinoma (PTC) is the most common histological subtype of thyroid cancer, accounting for 90% of new cases [3]. Surgery combined with thyroid

 doi:10.20944/preprints202603.1110.v1

stimulating hormone (TSH) suppression therapy or radioactive iodine therapy is the preferred treatment for PTC, and the prognosis of patients is usually good, but the rate of cervical lymph node metastasis is high, which may lead to local recurrence. Some patients may also experience distant metastasis and develop resistance to iodine-131, resulting in poor prognosis [4]. Therefore, how to identify specific PTC patients and adopt specific treatment methods for different patient conditions has become an important research topic at present.

The most direct consequence of aerobic glycolysis is the increase in intracellular and extracellular lactate concentrations [5]. The occurrence of aerobic glycolysis is attributed to the increased demand for ATP metabolism in proliferating cells (such as tumor cells) [6], and the accumulation of lactate in the microenvironment is a characteristic of inflammatory diseases and cancer [7]. Under normal circumstances, lactate is mainly converted into pyruvate through aerobic respiration and enters the mitochondria for further metabolism. However, in tumor cells, due to their high metabolic requirements and micro-oxic environment, anaerobic glycolysis significantly increases, leading to excessive lactate production [8]. The accumulation of lactate not only alters the metabolic characteristics of tumor cells but also promotes the invasiveness and metastatic ability of tumor cells by establishing a low pH environment [9]. Moreover, lactate has been proven to affect the function of immune cells in the tumor microenvironment, further promoting the development of cancer.

Lactate metabolism-related genes play a crucial role in various tumor types. For example, the expression of lactate dehydrogenase (LDH) and lactate transporter (LT) is closely related to the invasiveness and metastatic potential of tumors [10]. However, the specific mechanism of these genes in papillary thyroid carcinoma remains unclear.

Lactate may be related to the invasion and prognosis of thyroid cancer. Lactate can stimulate the migration, invasion and tumor angiogenesis of thyroid cancer cells, and may promote the invasion and metastasis process of tumors by regulating the activity of transcription factors and signaling pathways [11]. Lactate can also promote the formation of the immune escape network, allowing tumor cell proliferation to be uncontrolled [12]. The accumulation of lactate can induce the differentiation of myeloid-derived suppressor cells (MDSC), regulatory T cells (Treg), and tumor-associated macrophages (TAM), stimulating their biological activity, and subsequently secreting immunosuppressive factors, inhibiting the immune response of natural killer cells (NK cells) and T cells, helping tumor cells evade immune surveillance and acquire unlimited growth potential [13]. Therefore, lactate metabolism plays an important role in the occurrence and development of thyroid cancer (TC), and may become a new therapeutic target. Regulating the tumor acidification environment or interfering with lactate metabolism-related signaling pathways may help in the treatment of thyroid cancer [14].

This study screened and verified differentially expressed lactate metabolism-related genes (DE-LRGs), and finally determined TIMP1 and DPP4 as two significantly differentially expressed lactate metabolism-related genes as experimental genes. The relationship between these genes and lactate metabolism was explored, and the influence of gene expression on their occurrence and development was verified. Regression analysis was used to screen genes related to the prognosis of papillary thyroid carcinoma. Lasso regression analysis was used to obtain the optimal risk score value for each sample, for subsequent related analysis. Based on the median of the risk score, patients were divided into high-risk and low-risk groups, and the K-M curve was used for analysis. A prediction model was constructed based on these genes. This model demonstrated excellent predictive performance in both the training set and the internal validation set.

2. Materials and Methods

2.1. Research Subject

In this study, human derived papillary thyroid carcinoma cell lines, including TPC, IHH4, BPCAP, and normal thyroid cell line NTHY, were used as cells. All cells are from Fenghui Biotechnology Company.To ensure the optimal state of cells during cell culture and the

 doi:10.20944/preprints202603.1110.v1

reproducibility of experimental data, DMEM/1640 basic medium (45 ml), premium fetal bovine serum (5 ml), and penicillin streptomycin mixed antibiotics (P/S, 0.5ml) were used as the culture medium.

Identification Declaration: "All human cell lines have been identified through STR typing, and the identification reports are valid for three years." Pollution Control Declaration: "The cells used in the experiment have been tested and confirmed to be free of mycoplasma contamination."

The cells were cultured in a constant temperature incubator at 37 ℃ and 5% CO_2. To ensure that the cells used in the experiment have stable biological characteristics, all experiments were conducted using cells that have been passaged for 3-8 generations.

2.2. Differential Expression LRGs Screening

The RNA-seq dataset was statistically analyzed using the limma package to identify the differentially expressed genes between the healthy and diseased samples. The criteria for selecting the differentially expressed genes were: adj.P.Val < 0.05 & |logFC| > 0.58. Through the analysis of the expression profile of TCGA- papillary thyroid carcinoma, the differentially expressed genes related to PTC were screened out. The obtained results were intersected with the genes related to lactate metabolism to obtain the differentially expressed LRGs in PTC.

2.3. Obtaining Prognostic Related Genes and Constructing Prediction Models

Select prognostic related genes and use Lasso regression to further construct a prognostic related model.

2.4. Exploration of Specific Signal Mechanisms in Prognostic Models

We use GSVA and GSEA enrichment analysis for exploration. GSVA enrichment analysis downloaded genes from the Molecular signatures database, and GSVA performed gene set enrichment analysis (GSEA) on the expression profile of PTC patients; http://www.broadinstitute.org/gsea ） To search for DE LRGs between high-risk and low-risk groups of patients.

2.5. Exploring the Clinical Predictive Value of Multi Omics Models

The CIBERSORT algorithm was used to analyze RNA seq data from different subgroups of papillary thyroid carcinoma patients, in order to infer the relative proportions of 22 immune infiltrating cells. Pearson correlation analysis was performed on risk score values and immune cell content, and P<0.05 was considered statistically significant.

Based on the largest pharmacogenomics database (GDSC Cancer Drug Sensitivity Genomics Database, https://www.cancerrxgene.org/ ） We use the R software package "pRRophetic" to predict the chemotherapy sensitivity of each tumor sample; Use regression to obtain IC50 estimates for each specific chemotherapy drug treatment, and conduct 10 cross validation tests on the GDSC training set to verify the regression and prediction accuracy. All parameters have been set to default values.

2.6. Screening of Candidate Experimental Genes

The lactate metabolism-related genes with differential expression were sorted based on the fold change value (logFC) and expression abundance, resulting in 12 candidate genes (logFC > 2, sorted by expression abundance from high to low). Combining the search results of these 12 genes in tumors and lactate metabolism, TIMP1, SLPI, GDF15, TGFA, LCN2, and DPP4, which have relatively high correlations, were selected as the experimental genes.

2.7. qRT-PCR

The total RNA was extracted using TRIzol reagent. 2 μg of the total RNA was used for reverse transcription. The experiment was conducted using the Bio-Rad CFX96 system, and the expression

 doi:10.20944/preprints202603.1110.v1

of mRNA was calculated. The relative expression was evaluated using the ΔΔCt value. All primers were purchased from Integrated DNA Technologies (USA).

2.8. Transwell

Add 100 microliters of cell suspension to the Transwell chamber, and add 600 microliters of DMEM medium containing 20% serum to the 24-well plate. When inoculating the culture plate, be sure to avoid forming bubbles. Set up the control group (TIMP1), TIMP1 knockdown group 1, TIMP1 knockdown group 2, DPP4 knockdown group 1, DPP4 knockdown group 2, and control group (DPP4). Each group has 3 replicate samples. Use two papillary thyroid cancer cell lines (TPC, IHH4) for the experiment. After inoculation, culture the cells routinely for 24 hours. Wash with PBS and stain with crystal violet solution for a few minutes. Use a cotton swab to wipe off the migrating cells on the upper layer, then wash with PBS three times. Observe the cells in each well under a fluorescence inverted microscope, count them, take the average value, and select the images with better migration effects for photography and statistical analysis.

2.9. CCK8

A certain number of cells were inoculated into a 96-well cell culture plate (2000 cells per well for the cell proliferation experiment). The plate was placed in a incubator for one day. Meanwhile, a blank group was set up (the blank group contained only the culture medium and CCK-8 Solution). The 96-well cell culture plate was placed in a cell incubator (37℃, 5% CO2) for incubation for an appropriate period of time.The samples of the first day of the detection group and the blank group were added to each well of the 96-well plate (20 μL of CCK-8 Solution per well), and incubated at 37℃ for 1 hour. After incubation, the absorbance values were measured using an enzyme-labeled instrument. The above operation was repeated at 24h, 48h, and 72h. After 24h, the cells in the 96-well plate were changed to another one.The absorbance at 450nm of cells in each of the six groups (NC group, TIMP1 knockdown group 1, TIMP1 knockdown group 2, NC group, DPP4 knockdown group 1, DPP4 knockdown group 2) on days 1, 2, 3, and 4 was recorded. When the differences between the duplicate wells were too large, they were excluded. The average values of the duplicate wells were taken and recorded for plotting.

2.10. Lactic Acid Test

Set up the blank group, the standard group and the sample group. In the blank group, add 5 microliters of deionized water and 200 microliters of the detection buffer, mix well, incubate at 37°C for 5 minutes, and then add 40 microliters of the enzyme solution; in the standard group, add 5 microliters of the lactic acid standard solution and 200 microliters of the detection buffer, mix well, incubate at 37°C for 5 minutes, and then add 40 microliters of the enzyme solution; in the sample group, add 5 microliters of the sample and 200 microliters of the detection buffer, mix well, incubate at 37°C for 5 minutes, and then add 40 microliters of the enzyme solution. After all groups are mixed, measure the absorbance at 546 nanometers using a spectrophotometer and calculate the lactic acid content.

2.11. Lactate Dehydrogenase Test

According to the experimental requirements, different groups were set up. The cells were cultured for one day, washed with PBS, 120 μL of cell lysis solution was added, incubated in the incubator for a few minutes, centrifuged after the incubation, and then 80 μL of the solution was added to the pre-prepared 96-well plate. Prepare an appropriate amount of LDH detection working solution according to the number of the samples to be tested. Conduct experiments on approximately 50 samples, adding 1 ml of reaction substrate, 100 μL of INT solution, 150 μL of enzyme solution and 2.75 ml of reaction buffer (total volume 4 ml). Add the LDH detection working solution to the sample

 doi:10.20944/preprints202603.1110.v1

wells, 80 μL per well, and gently shake to mix. Place the cells in a light-protected incubator for a few minutes, then detect the absorbance of LDH.

2.12. Statistical Analysis

Data analysis was performed using GraphPad Prism (version 10.0.2) statistical software. All experimental data were expressed as mean ± standard deviation (x ± s), and pairwise comparisons between groups were conducted using the LSD-t test, while comparisons among multiple groups were performed using one-way analysis of variance. A P value < 0.05 indicated statistically significant differences. Survival curves were generated using the kaplan-meier method and compared using the log-rank test. This analysis was conducted using R language (version 4.0). All statistical tests were two-sided, and a P value < 0.05 was considered statistically significant.

3. Results

3.1. Analysis of mRNA Expression Levels (Patients vs. Controls)

We used the limma package to statistically analyze the RNA-seq dataset to identify the differentially expressed genes between the diseased and healthy samples. The criteria for selecting the differentially expressed genes were: adj.P.Val < 0.05 & |logFC| > 0.58. Through the analysis of the expression profile of TCGA- papillary thyroid carcinoma, a total of 2,290 thyroid papillary carcinoma-related differentially expressed genes were screened out, including 1,213 up-regulated genes and 1,077 down-regulated genes (Figure 1a-1b). Among them, there were 222 genes related to lactate metabolism (Figure 1c).

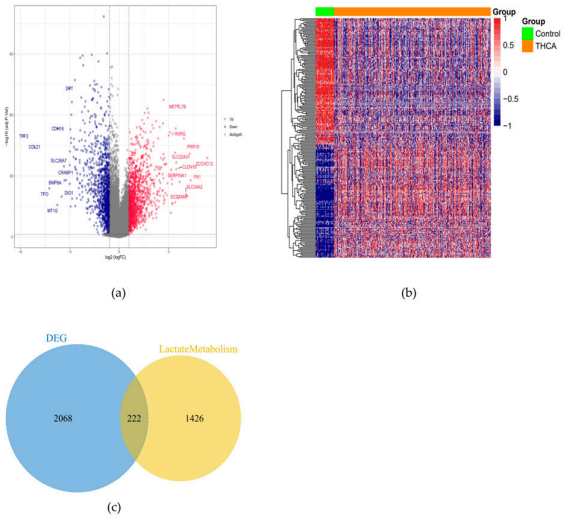


Figure 1. The mRNA expression level and genes related to lactate metabolism:(a)2,290 differentially expressed genes related to papillary thyroid carcinoma: 1,213 up-regulated genes and 1,077 down-regulated genes.(b)Heat map distribution.(c)After taking the intersection of the differentially expressed genes of papillary thyroid carcinoma and the genes related to lactate metabolism, 222 DE-LRGs were selected.

3.2. DE-LRGs Undergo Consistent Cluster Analysis

The expression consistency of 222 DE-LRGs in the PTC cohort was analyzed using the R package ConsensusClusterPlus. It was found that their consistency was the highest when the clustering number k was 3(Figure 2a-2b). The survival differences among the three subtypes were evaluated

 doi:10.20944/preprints202603.1110.v1

using the K-M curve, and the prognostic differences of the three subtypes were explored. The results showed that compared with C1 and C3, C2 had a better survival possibility (Figure 2c-2d). This indicates that by combining the expression of genes related to tissue lactate metabolism, we can predict the population with a higher risk of recurrence.

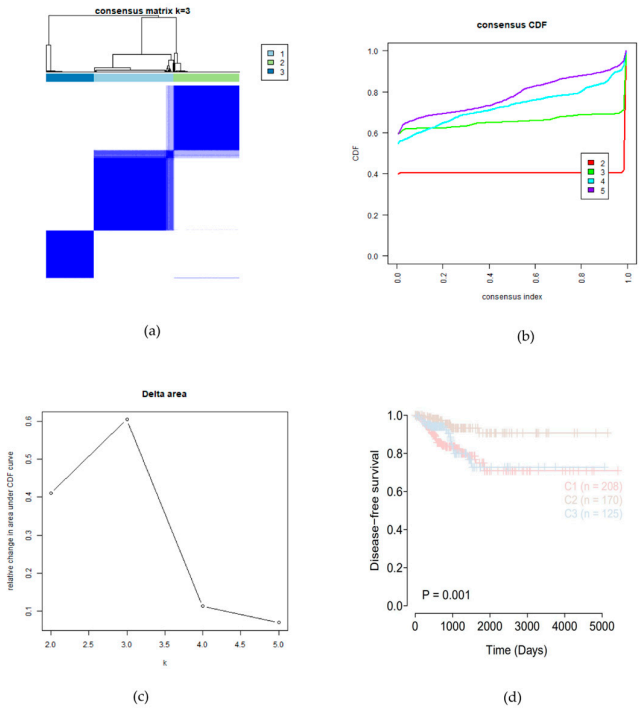
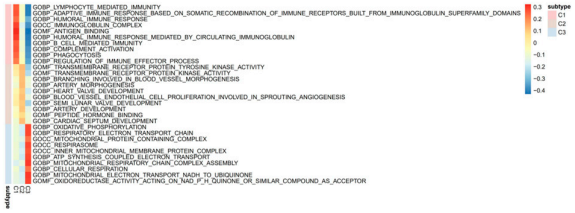


Figure 2. The results of the consistency clustering analysis show that, compared with C1 and C3, C2 has a higher survival probability; *P*<0.001.

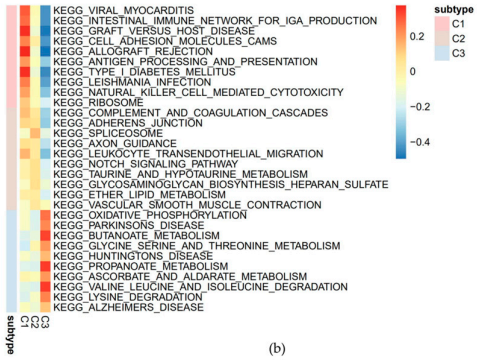
3.3. Evaluation of Subtype Molecular Characteristics

This study further conducted quantitative analysis of GO and KEGG processes using the ssGSEA algorithm, revealing that there were significant differences in multiple related pathways among the subgroups. Among them, in the GO analysis, the pathways with higher C2 subtype scores mainly included transmembrane receptor protein tyrosine kinase activity (transmembrane receptor protein tyrosine kinase activity), transmembrane receptor protein kinase activity (transmembrane receptor protein kinase activity), and branching involved in blood vessel morphogenesis (signal pathways such as transmembrane receptor protein tyrosine kinase activity, transmembrane receptor protein kinase activity, and branching involved in blood vessel morphogenesis, as shown in Figure 3a). In the KEGG analysis, the pathways with higher C2 subtype scores mainly included notch signaling pathway (Notch signaling pathway), taurine and hypotaurine metabolism (taurine and hypotaurine metabolism), and glycosaminoglycan biosynthesis heparan sulfate (glycosaminoglycan biosynthesis with heparan sulfate, as shown in Figure 3b).

 doi:10.20944/preprints202603.1110.v1



(a)



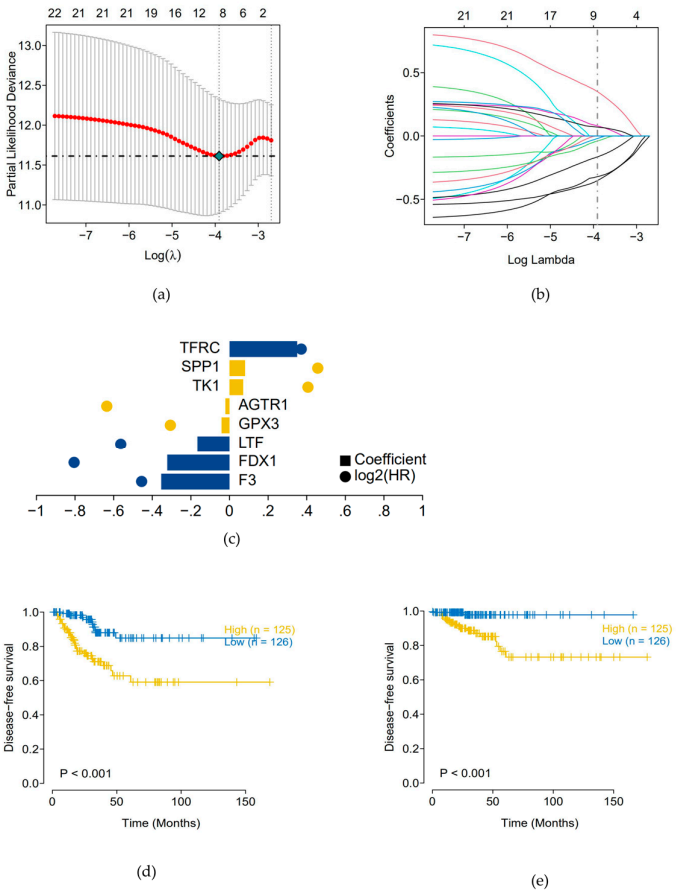
(b)

Figure 3. Evaluation results of subtype molecular characteristics:(a)Pathways with higher scores for C2 subtype in GO analysis.(b)Pathways with higher scores for the C2 subtype in KEGG analysis.

3.4. Obtain Prognostic-Related Genes and Construct a Prediction Model

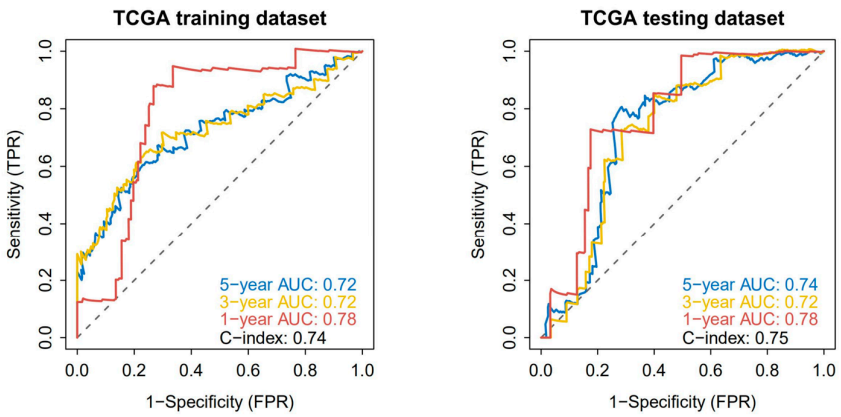
Clinical information of PTC patients was collected. Through Cox univariate regression using the DE-LRGS gene set, 22 prognostic-related genes were selected. Using the lasso regression feature selection algorithm, characteristic genes in thyroid papillary carcinoma were screened out (Figure 4a-4c). The TCGA patients were randomly divided into training set and internal validation set at a 1:1 ratio. After lasso regression analysis, the best risk score value corresponding to each sample was obtained for subsequent related analysis (Risk Score = F3 x (-0.35240308247203) + FDX1 x (-0.321572965907877) + LTF x (-0.165176924477088) + GPX3 x (-0.0399881453829196) + AGTR1 x (-0.0201482218613706) + TK1 x 0.0699517206164314 + SPP1 x 0.0797818398286089 + TFRC x 0.350195792033765). Patients were divided into high-risk group and low-risk group based on the median of risk score, and Kaplan-Meier curves were used for analysis. The DFS of the high-risk group in the training set and test set was significantly lower than that of the low-risk group (Figures 4d-4e). In addition, the ROC curve results showed that the AUC values of the training set and test set at 1 year, 3 years, and 5 years were all not less than 0.7 (Figure 5a-5b), indicating that the predictive effect of the model was good.

 doi:10.20944/preprints202603.1110.v1



(a) (b) (c) (d) (e)

Figure 4. The screening results of characteristic genes in papillary thyroid carcinoma and the DFS of the training set and test set:(a)Prognostic lasso regression analysis results.(b)Prognostic lasso variable trajectory results.(c)Analysis of screening results by Lasso regression algorithm.(d)The results of Kaplan-Meier curve analysis showed that the DFS of the high-risk group in the training set was significantly lower than that of the low-risk group; *P*<0.001.(e)The results of Kaplan-Meier curve analysis showed that the DFS of the high-risk group in the test set was significantly lower than that of the low-risk group; *P*<0.001.



(a) (b)

Figure 5. Model evaluation results:(a)The ROC curve results show that the AUC values calculated for the three periods (1 year, 3 years, and 5 years) in the training set are greater than 0.7, indicating that the model has good validation

 doi:10.20944/preprints202603.1110.v1

efficacy.(b)The results of the ROC curve showed that the AUC values calculated for the 1-year, 3-year, and 5-year periods in the test set were greater than 0.7, indicating that the model has good validation performance.

3.5. Constructing the Nomogram and the Calibration Curve

The clinical information and risk scores of patients in the high-risk and low-risk groups were integrated. The results of the regression analysis were presented in the form of a nomogram. The results of the logistic regression analysis indicated that in all of our samples, the clinical indicator values of age, M, and riskscore contributed to multiple scoring processes. The riskscore value and the distribution of the clinical indicator age obtained through model analysis had a higher contribution in the scoring process of the prediction analysis (Figure 6). The prediction analysis for the three-year and five-year periods of thyroid papillary carcinoma revealed that the predicted DFS was in good agreement with the observed DFS (Figure 7a-7c). This indicates that the model has certain predictive value. Combined with the general clinical characteristics of the patients, the expression level of lactate metabolism genes, the recurrence risk of the patients can be predicted.

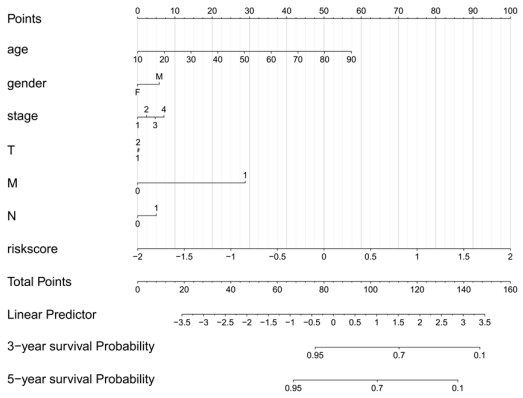


Figure 6. The clinical indicator values of thyroid papillary carcinoma, including age, M and riskscore, all contribute to the distribution in multiple scoring processes.

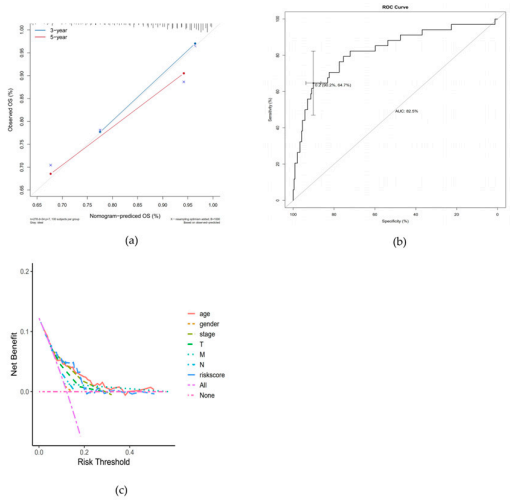


Figure 7. The three-year and five-year survival analysis results of papillary thyroid cancer:The predictive analysis conducted for the three-year and five-year periods of papillary thyroid carcinoma revealed that the predicted DFS was in good agreement with the observed DFS.

 doi:10.20944/preprints202603.1110.v1

3.6. The Results of the Signaling Pathways Involved in the Prognosis Model

The study explored the signaling pathways involved in different risk group models and initially identified the potential mechanisms by which the risk score affects the development of PTC. The GSVA analysis mainly enriched e2f targets, cholesterol homeostasis, mitotic spindle, etc. (Figure 8). Through GSEA analysis, it was found that many related pathways were significantly enriched. The GO-enriched pathways included chaperone-mediated autophagy, DNA replication initiation, etc.; the KEGG-enriched pathways included alanine aspartate and glutamate metabolism, DNA replication, etc. (Figure 9a-9b). For some of the highly significant pathways, they were presented in a concentrated manner. The results showed that the disturbances of these signaling pathways in patients with high and low risk groups might be associated with the prognosis of PTC patients.

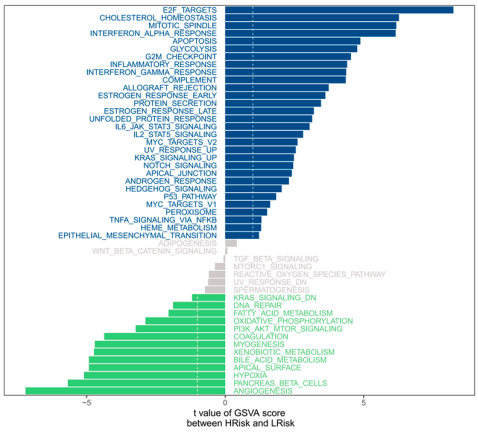


Figure 8. The GSVA analysis revealed that the signaling pathways involved in the different risk group models were mainly enriched in e2f targets, cholesterol homeostasis, and mitotic spindle.

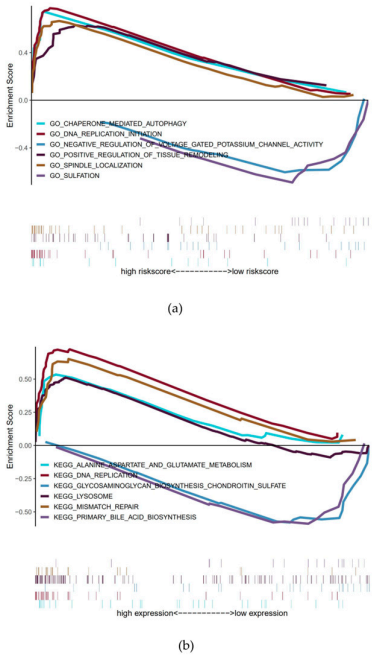


Figure 9. The results of GSEA GO analysis and GSEA KEGG analysis results:(a)Through GSEA analysis, it was found that many of the related pathways were significantly enriched. The pathways enriched by GO included chaperone-mediated autophagy and DNA replication initiation.(b)The pathways enriched by KEGG include alanine aspartate and glutamate metabolism, and DNA replication.

 doi:10.20944/preprints202603.1110.v1

3.7. Exploring the Clinical Predictive Value of the Model

By analyzing the relationship between the risk score and tumor immune infiltration, we further explored the potential molecular mechanism by which the risk score affects the progression of papillary thyroid carcinoma. The results showed that the distribution of immune levels of different immune factors in the samples was not completely consistent (Figure 10). There were multiple significant correlations among immune factors (Figure 11). Compared with the high-risk group, the levels of immune factors such as NK cells activated were significantly higher in the low-risk group samples, while the levels of immune factors such as naive B cells, regulatory T cells (Tregs), etc. were significantly lower (Figure 12). The risk score was significantly positively correlated with T cells regulatory (Tregs), Macrophages M0, etc., and significantly negatively correlated with T cells CD8, NK cells activated, etc. (Figure 13). Based on the drug sensitivity data from the GDSC database, we used the R package "pRRophetic" to predict the chemotherapy sensitivity of each tumor sample and further explored the relationship between the risk score and the sensitivity to common chemotherapy drugs. The results showed that the level of the risk score was significantly correlated with the sensitivity of patients to drugs such as Axitinib, Bicalutamide, Bortezomib, Bosutinib, Cisplatin, and Cyclopamine (Figure 14). At the same time, the distribution of mutation types in the high and low expression groups of the patient samples was not completely consistent (Figure 15). At the same time, the level of tumor mutational burden (TMB) also had significant differences between the groups (Figure 16). Through the analysis of tumor immune dysfunction and rejection, it was found that there were differences in the high-risk and low-risk groups, and Responder, Exclusion, etc. showed significant differences between the high-risk and low-risk groups (Figure 17a-17b). The above results indicate that lactate metabolism may regulate the malignancy of tissues through complex immune regulatory mechanisms, thereby influencing the recurrence risk of patients.

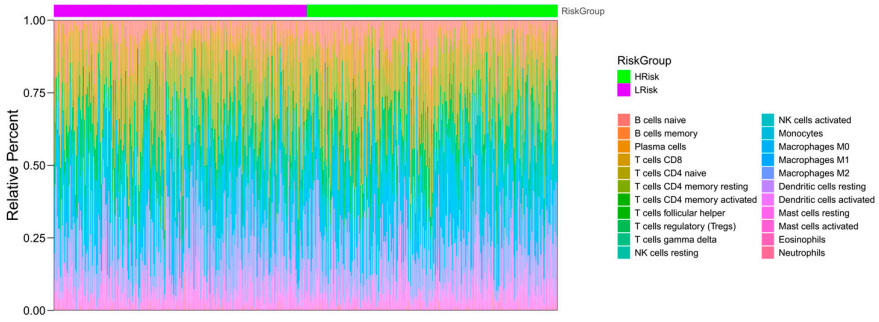


Figure 10. The distribution of immune levels of different immune factors in samples.

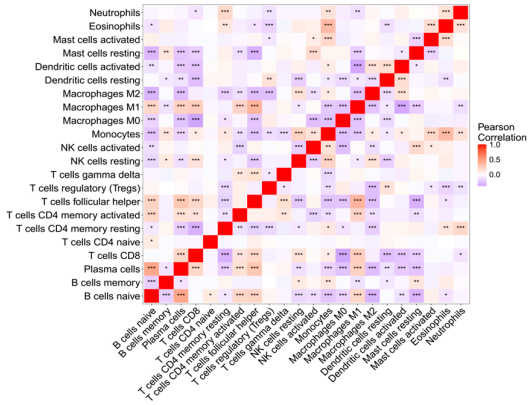


Figure 11. The interrelationship among immune factors on;$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

 doi:10.20944/preprints202603.1110.v1

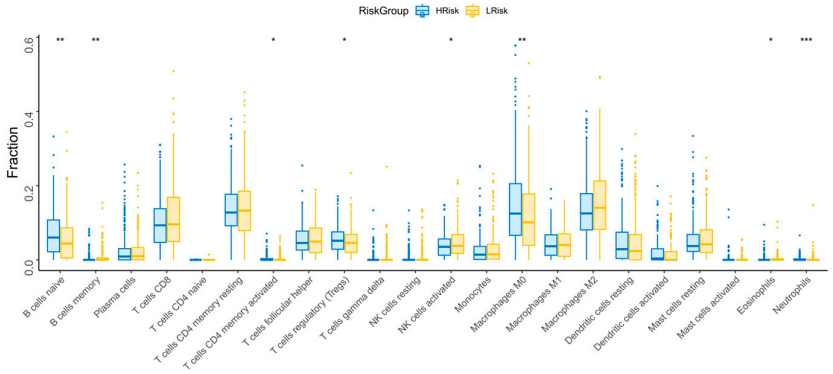


Figure 12. Immune factor levels in high-risk and low-risk groups;*P<0.05, **P<0.01 , ***P<0.001.

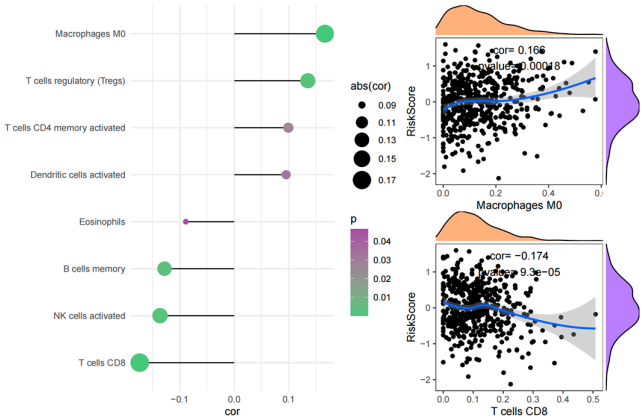


Figure 13. The relationship between risk score and immune cells;P<0.001.

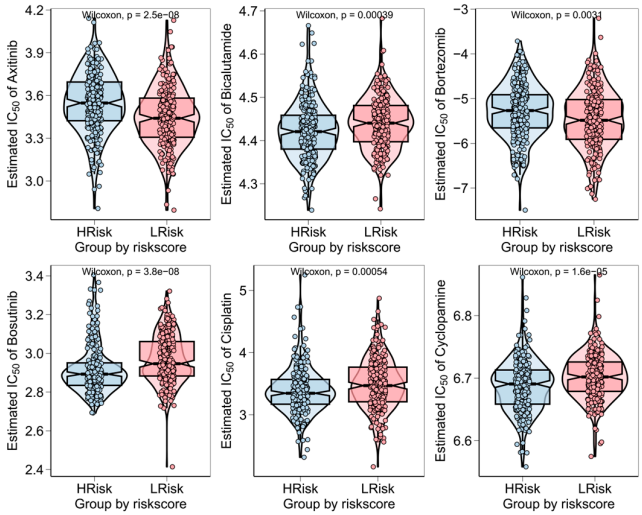


Figure 14. The level of risk assessment is significantly correlated with the patients' sensitivity to drugs such as Axitinib, Bicalutamide, Bortezomib, Bosutinib, Cisplatin and Cyclopamine.

 doi:10.20944/preprints202603.1110.v1

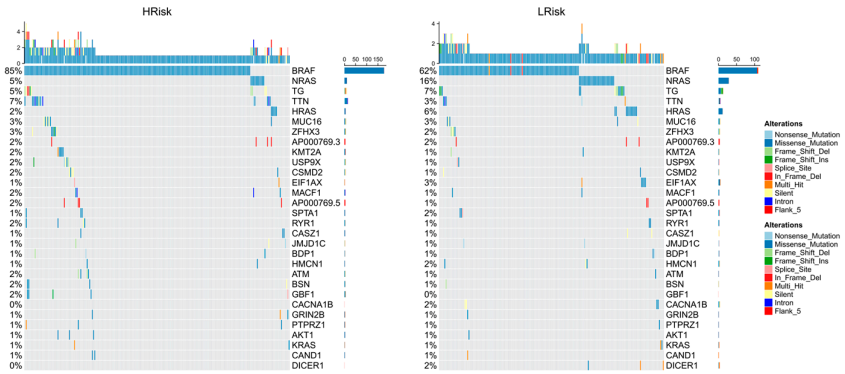


Figure 15. Distribution of mutation types in high-risk and low-risk groups results.

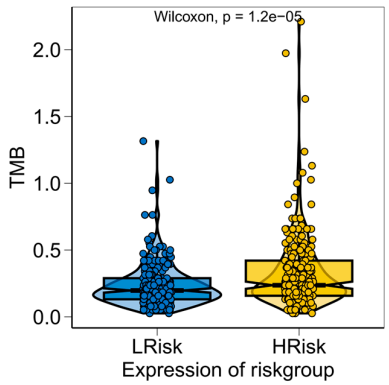


Figure 16. The results showing the difference in tumor mutational burden between the high-risk group and the low-risk group indicated that the tumor mutational burden in the high-risk group was lower than that in the low-risk group.

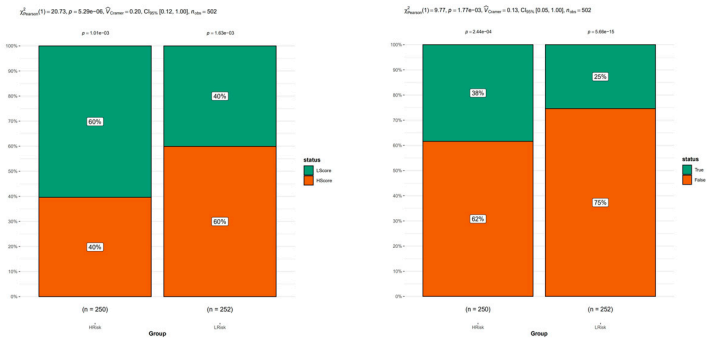


Figure 17. Differences in tumor immune function between high-risk and low-risk groups:There were significant differences in variables such as Responder and Exclusion between the high-risk and low-risk groups.

3.8. Selection of Alternative LRGs Genes

The above studies have confirmed that genes related to lactate metabolism play an important role in PTC, participating in a complex regulatory network. Different gene expression patterns can be used to screen patients with a higher risk of recurrence. Based on bioinformatics research, in order to further verify the role of lactate metabolism genes in PTC, we screened the corresponding genes

 doi:10.20944/preprints202603.1110.v1

for functional validation studies. The expression information of 222 LRGs was sorted according to LogFC, and the genes with more obvious differential expression were selected as candidate genes for further screening (Figure 18). The gene + lactate metabolism + cancer was searched one by one on PubMed, and it was found that in other tumors, studies related to lactate metabolism were more numerous and not involved in thyroid papillary carcinoma. There were 6 genes that were not studied in thyroid papillary carcinoma but were related to lactate metabolism in other tumors, namely TIMP1, DPP4, SLPI, GDF15, TGFA, and LCN2. These genes were selected as pre-selected genes for qRT-PCR verification.

	Symbol	logFC	AveExpr	t	P.Value	adj.P.Val	B
2	FN1	4.343475609484	7.7598487040	12.16115413096	2.2800725	1.4648418	57.0940668969483
3	SERPINA1	3.66695527449	7.0871403858	11.98609970524	1.2347435	7.4951079	55.4138068806966
4	GDF15	3.40828081831	4.5897088794	15.29440220495	2.1309642	4.4278606	89.2722868136657
5	SFTPB	3.13516918253	5.5274762417	7.6441240644549	1.1626227	1.3127306	19.1952992099345
6	LCN2	2.74979760951	3.3366116515	9.7148664532	1.0007089	2.8051477	35.0424369234019
7	DPP4	2.65612609560	2.8480264419	14.53399715324	7.4408871	1.1338194	81.1392244533415
8	THRSP	2.48171360394	3.3627024596	13.47868951560	4.4839718	4.6504187	70.1729524812787
9	TIMP1	2.47545522870	7.9976217184	11.15729219693	3.0117004	1.3778008	47.6604064365618
10	NPC2	2.24170917462	8.6452831247	18.60806058757	1.4605620	1.1420634	126.411481012261
11	TGFA	2.07194728191	3.9364170719	16.01577894491	7.9579164	2.1496141	97.1438400174671
12	SLPI	2.06171675968	5.7008644877	16.65580312230	6.7114581	7.1183619	12.7114504982987
13	APOE	1.96822578138	6.9147794924	7.3405067333627	7.5086080	9.7746780	17.1243332061374

Figure 18. After conducting a detailed search on 222 DE-LRGs, it was found that there were 6 genes that have been extensively studied in other tumors and are related to lactate metabolism, but have not been investigated in thyroid papillary carcinoma. These genes are TIMP1, DPP4, SLPI, GDF15, TGFA, and LCN2.

3.9. The Results of qRT-PCR Verification

The repeated qRT-PCR experiment verification results showed that compared with normal thyroid cells, TIMP1 and DPP4 were upregulated in the thyroid papillary carcinoma cell lines TPC, IHH4, and BPCAP, which was consistent with the results of bioinformatics screening; SLPI, GDF15, TGFA, and LCN2 were downregulated in the thyroid papillary carcinoma cell lines, which was inconsistent with the results of bioinformatics analysis, so they were excluded (Figure 19).

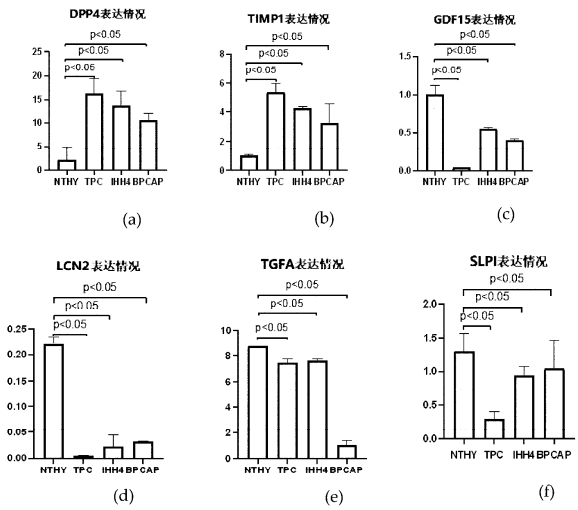


Figure 19. The expression of genes in thyroid cell lines and papillary thyroid carcinoma cell lines; *P*<0.05:The qRT-PCR results showed that compared with the expression in normal thyroid cell lines, TIMP1 and DPP4 were upregulated in the thyroid papillary carcinoma cell lines TPC, IHH4, and BPCAP; GDF15, LCN2, SLP1, and TGFA were also upregulated in the thyroid papillary carcinoma cell lines.

 doi:10.20944/preprints202603.1110.v1

3.10. The Effects of TIMP1 and DPP4 on Lactate Metabolism

Four thyroid cell lines were cultured. After 24 hours, the cell culture supernatant was centrifuged at 2500 rpm for 10 minutes. The sample was loaded onto a 96-well plate using the Servicebio lactic acid detection kit. The OD value at 546 nm was measured by spectrophotometry to calculate the lactate content. The results showed that compared with the normal thyroid cell line NTHY, the thyroid papillary carcinoma cell lines (BPCAP, TPC, IHH4) had stronger lactate metabolism and excessive accumulation of lactate (Figure 20).

The Servicebio Lactate Dehydrogenase Cytotoxicity Detection Kit can be used for cell LDH detection. Measuring the LDH content can indirectly reflect the strength of lactate metabolism. The test results show that compared with normal thyroid cell lines, the LDH content in thyroid papillary carcinoma cell lines is higher, and the lactate metabolism is relatively stronger. (Figure 21)

Compared with the control group, when the expressions of TIMP1 and DPP4 were downregulated, the lactate metabolism significantly decreased and the ability to accumulate lactate weakened. (Figure 22a-22b)

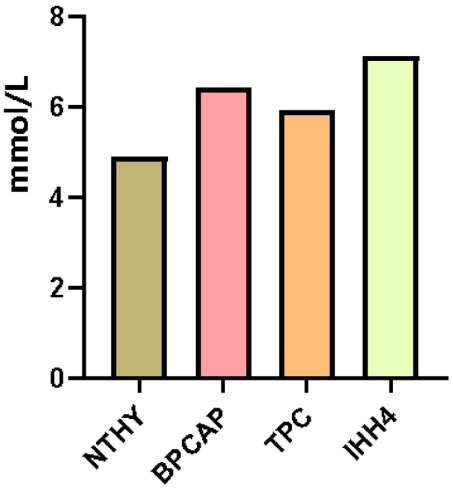


Figure 20. Lactic acid content of NTHY、BPCAP、TPC、IHH4 cell lines; *P*<0.05:Compared with the normal thyroid cell line NTHY, the thyroid papillary carcinoma cell lines (BPCAP, TPC, IHH4) have a stronger lactate metabolism and excessive lactate accumulation.

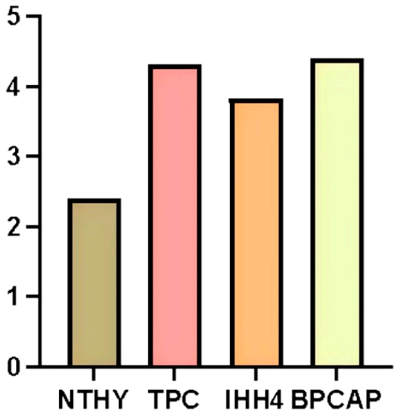


Figure 21. LDH activity of NTHY, TPC, IHH4 and BPCPA cell lines; *P*<0.05:Compared with normal thyroid cell lines, the thyroid papillary carcinoma cell lines have a higher LDH content and a stronger lactate metabolism.

 doi:10.20944/preprints202603.1110.v1

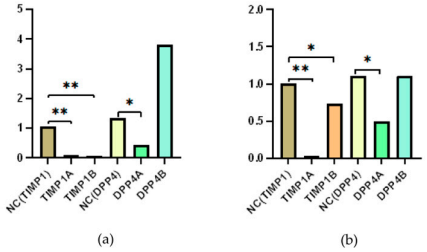


Figure 22. The expression of the gene after transfection in TPC and IHH4 cells:(a)The results showed that in the thyroid papillary carcinoma cell line TPC, compared with the TIMP1B group and the DPP4B group, the TIMP1A group and the DPP4B group performed better.(b)The results showed that in the thyroid papillary carcinoma cell line IHH4, compared with the TIMP1B group and the DPP4B group, the TIMP1A group and the DPP4B group performed better.

3.11. The Effects of TIMP1 and DPP4 on the Proliferation and Metastasis of PTC

To further explore the impact of genes on cell proliferation and migration through regulating lactate metabolism, we conducted transfection to down-regulate TIMP1 and DPP4 and then verified it using qRT-PCR. The results showed that in the thyroid papillary carcinoma cell lines TPC and IHH4, compared with the TIMP1B group and DPP4B group, the TIMP1A group and DPP4B group performed better for subsequent experiments. (Figure 23)

Compared with the control group (NC), when the expressions of TIMP1 and DPP4 were downregulated, the proliferation ability of TPC and IHH4 cell lines was weakened (Figure 24); when the expressions of TIMP1 and DPP4 were downregulated, the migration ability of TPC and IHH4 cell lines was also weakened (Figure 25).

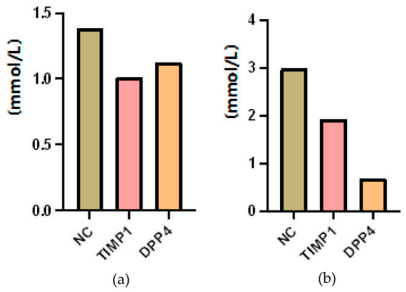


Figure 23. Changes in lactate content after down-regulation of TIMP1 and DPP4:(a)After the downregulation of TIMP1 and DPP4 in the TPC cell line, the lactate content decreased.(b)After the downregulation of TIMP1 and DPP4 in the IHH4 cell line, the lactate content decreased.

 doi:10.20944/preprints202603.1110.v1

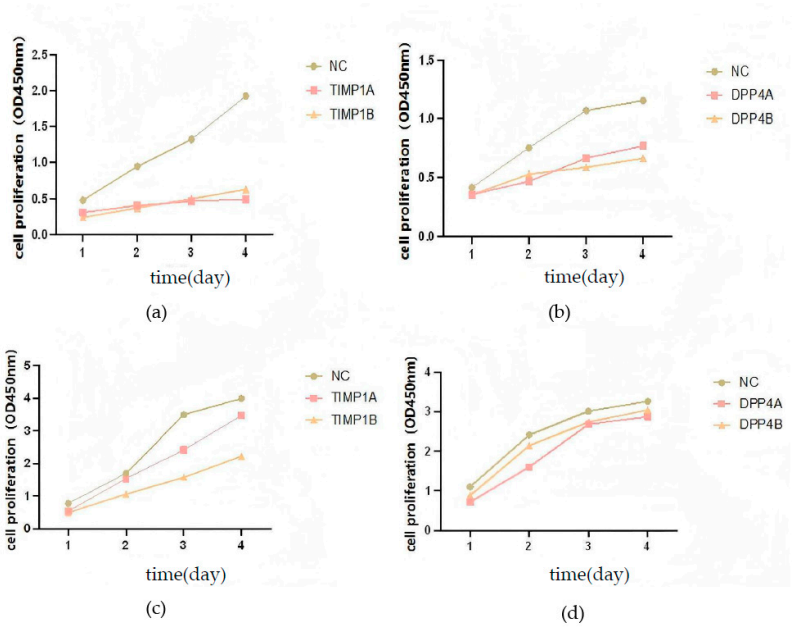


Figure 24. Down-regulation of TIMP1 and DPP4 leads to changes in cell proliferation:(a)The changes in cell proliferation over time after the downregulation of TIMP1 expression in the TPC cell line(b)The changes in cell proliferation over time after the downregulation of DPP4 expression in the TPC cell line.(c)The changes in cell proliferation over time after the downregulation of TIMP1 expression in the IHH4 cell line.(d)The changes in cell proliferation over time after the downregulation of DPP4 expression in the IHH4 cell line.

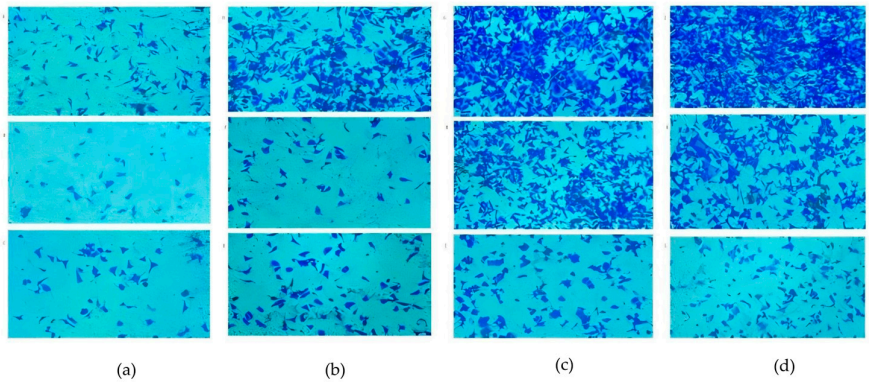


Figure 25. The changes in cell migration ability after down-regulating TIMP1 and DPP4:(a)After down-regulating the expression of TIMP1 in the TPC cell line using different si-RNA, the cell migration ability was significantly weakened.(b)After down-regulating the expression of TIMP1 in the IHH4 cell line using different si-RNA, the cell migration ability was significantly weakened.(c)After down-regulating the expression of DPP4 in the TPC cell line using different si-RNA, the cell migration ability was significantly weakened.(d)After down-regulating the expression of DPP4 in the IHH4 cell line using different si-RNA, the cell migration ability was significantly weakened.

4. Discussion

Existing studies have confirmed that genes related to lactic acid metabolism play a significant role in tumor prognosis [15–19]. For instance, in lung adenocarcinoma, it has been demonstrated that the lactic acid metabolism-related gene KRT81 plays a crucial role in the occurrence and development of lung adenocarcinoma. By establishing a nude mouse xenograft model and injecting A549 cells transfected with siNC and siRNA2, the tumor changes were closely monitored. The results confirmed

 doi:10.20944/preprints202603.1110.v1

that KRT81 plays an important role in the progression of lung adenocarcinoma [20]. Although extensive bioinformatics analysis and basic experimental research on lactic acid metabolism-related genes have been conducted in many tumors, studies on papillary thyroid carcinoma (PTC) are relatively scarce. The impact of lactic acid metabolism-related genes on lactic acid metabolism in PTC and their role in the proliferation and metastasis of PTC still require further investigation. Therefore, taking lactic acid metabolism-related genes as the entry point, combined with bioinformatics analysis, and through relevant basic cell experiments and multi-omics related analyses to prove the influence of lactic acid metabolism-related genes on the occurrence and development of PTC, as well as their impact on the tumor microenvironment, drug sensitivity, immune response, and disease-free survival of PTC patients, is particularly important.

The immune system plays a crucial role in preventing cancer and in the occurrence and development of cancer. Recent studies on immune infiltration in PTC have made many significant advances, especially in the research of major immune cells such as B cells and NK cells [21–26]. It has been found that these tumor-promoting immune cells are massively recruited in PTC and exhibit a tumor-promoting immune microenvironment phenotype [27–29]. This indicates that PTC can manipulate some immune cells, such as M2 macrophages, regulatory T cells, monocytes, neutrophils, dendritic cells, mast cells, and M0 macrophages, to suppress the body's immune response to itself, which can also be called "damage", ultimately enabling the tumor to evade immune surveillance and immune system attacks [30]. By analyzing the relationship between risk scores and tumor immune infiltration, we explored the potential molecular mechanisms by which risk scores affect the progression of papillary thyroid carcinoma. The results showed that the distribution of immune factors in different samples was not completely consistent. There were multiple significant correlations among immune factors.

In tumor-related research, the construction of predictive models has become an important task. The construction of predictive models has far-reaching and multi-level impacts, capable of integrating medical data from databases and serving as a tool for guiding clinical decision-making and scientific research. By constructing a predictive model based on disease-free survival, the aim is to predict the sensitivity of PTC patients to different drugs, tumor mutation burden, and immune treatment response, and to make preliminary predictions of the 1-year, 3-year, and 5-year disease-free survival of patients.

Although this study has demonstrated that genes related to lactate metabolism can serve as markers for predicting the disease-free survival period of PTC. The levels of immune cells, tumor microenvironment, and tumor burden may be involved in the pathogenesis of PTC, and it has been confirmed that genes such as TIMP1 and DPP4 related to lactate metabolism can regulate the lactate metabolism and proliferation and metastasis of PTC. However, this study still has some shortcomings. Firstly, due to the lack of external datasets for thyroid papillary carcinoma in this study, the prognostic model was not re-verified using external datasets. Secondly, the specific signaling pathways involved in the effects of TIMP1 and DPP4 on the occurrence and development of PTC and their impact on lactate metabolism need to be further explored. Further downstream experiments and animal experiments are needed to conduct further research. In future studies, we will improve these issues.

5. Conclusions

This study conducted DE-LRGs gene screening and validation to ultimately determine TIMP1 and DPP4 as the two lactate metabolism-related genes with relatively significant differential expression as the experimental genes. It explored the relationship between genes and lactate metabolism, and verified the influence of gene expression and its impact on the occurrence and development of papillary thyroid carcinoma. The results showed that TIMP1 and DPP4 were highly expressed in PTC, and lactate metabolism was inhibited after down-regulating the expression of TIMP1 and DPP4 genes, and the proliferation and migration ability of PTC were weakened.

 doi:10.20944/preprints202603.1110.v1

Through clustering analysis of 222 DE-LRGs genes, it was found that the consistency was the highest when k=3. The survival differences between subtypes were evaluated using the K-M curve, and the prognostic differences of different molecular subtypes were explored. The results showed that compared with C1 and C3, C2 had a better survival possibility.

Through single-factor Cox regression analysis and Lasso regression analysis, the PTCA-related genes were screened out. The best risk score value corresponding to each sample was obtained through Lasso regression analysis for subsequent related analysis. The patients were divided into high-risk and low-risk groups based on the median of risk scores, and the analysis was conducted using the K-M curve. Based on these genes, a prediction model was constructed, which showed good predictive efficacy in the training set and internal validation set and could effectively predict the recurrence and metastasis of PTC patients. At the same time, the risk score was combined with clinical indicators through the nomogram diagram to provide a more intuitive and accurate reference basis for clinical decision-making.

Author Contributions: MSJ, BN and XFD designed thisstudy and revised the manuscript. MSJ wrote the manuscript.OYXW,JX analyzed and interpreted the data.FGD,WHH,GRT participated in TCGA and GEO data analysis. All the listed authors have participated actively in the study. All the authors have read and approved the final manuscript.

Funding: This study was supported by the National Natural Science Foundation of China(NSFC 82260585, Ning Bai), National Youth Science Foundation Project(Xiwu Ouyang ,82203834).

Data Availability Statement: This analysis compared the differentially expressed genes of the diseased condition from the TCGA database (https://portal.gdc.cancer.gov/), and the lactate metabolism-related gene set (Lactate Metabolism) was derived from the GeneCard database (https://www.genecards.org).The data for drug sensitivity analysis were obtained from the Pharmacogenomics Database (GDSC Cancer Drug Sensitivity Genomics Database, https://www.cancerrxgene.org/).

Acknowledgments: We thank the TCGA and GEO databases for their data support.

Conflicts of Interest: The authors have declared that no competing interest exists.

Abbreviations

The following abbreviations are used in this manuscript:

DE-LRG	Differential expression lacate metabolism-related gene
DFS	Disease free survival
GEO	Gene Expression Omnibus
GO	Gene ontology
GSVA	Gene Set Variation Analysis
GSEA	Gene Set Enrichment Analysis
KEGG	Kyoto Genome Encyclopedia
LDH	lactate dehydrogenase
LMRGS	Lactate metabolism-related genes
LT	lactate transporter
MDSC	Myeloid-derived suppressor cells
PTC	Papillary Thyroid Carcinoma
TAM	Tumour-associated macrophages
TC	Thyroid Cancer
TCGA	The Cancer Genome Atlas

 doi:10.20944/preprints202603.1110.v1

TME	Tumor microenvironment
THCA	Thyroid Carcinoma

References

1. Sung H, Ferlay J, Siegel RL,et al. Global cancer statistics 2020:GLOBOCANestimates of incidence and mortality worldwide for 36 cancers in 185 countries[J]. CA Cancer J Clin. 2021,71:209–49.
2. Seib CD, Sosa JA. Evolving understanding of the epidemiology of thyroid canc-er[J].Endocrinol Metab Clin North Am. 2019,48:23–35.
3. Yixian Wang, Xin Li, Qingwei Gang, et al. Pathomics and single-cell analysis of papillary thyroid carcinoma reveal the pro-metastatic influence of cancer-associated fibroblasts[J]. B MC CANCER. 2024, Jun 10;24(1):710.
4. Minqi Xia, Shuo Wang, Li Wang, et al. The role of lactate metabolism-related LncRNAs i-n the prognosis, mutation, and tumor microenvironment of papillary thyroid cancer[J]. Frontiers in Endocrinology. 21 March 2023
5. Liberti, Locasale. The Warburg effect: How does it benefit cancer cells?[J]. Tren-ds Biochem. Sci. 2016,41, 211–218.
6. Teng R. HSP60 silencing promotes Warburg-like phenotypes and switches the mi-tochondri-al function from ATP production to biosynthesis in ccRCC cells[J]. Redox Biol. 2019, 24,101–218.
7. Certo M.Lactate modulation of immune responses in inflammatory versus tumo-ur microenvironments[J]. Nat. Rev. Immunol. 2021, 21, 151–161.
8. Fatemeh K,Moloud P, Masoumeh S, et al. Cancer Management and Research[J].Cell Meta. 2019, 11 1829–1841.
9. 张慧芬,曾志军,周艳红,等.乳酸代谢—肿瘤治疗新靶点[J].生物化学与生物物　理进展,2021 ,48: 02.
10. Fatemeh K,Moloud P, Masoumeh S, et al. Cancer Management and Research[J]. Cell Meta.2019, 11 1829–1841.
11. Yuanyuan Zhang, Zhao Zhai, Jiali Duan, et al. Lactate: The Mediator of Metab-olism and Immunosuppression[J].Frontiers in Endocrinology.2022:13:901495
12. Dichtl S, Lindenthal L, Zeitler L, et al. Lactate and IL6 Define Separable Path-s of Inflammatory Metabolic Adaptation[J]. SciAdv. 2021; 726:3505.
13. Brand A, Singer K, Koehl GE, Kolitzus M, Schoenhammer G, Thiel A,et al. L-DHA-Associated Lactic Acid Production Blunts Tumor Immunosurveillance byTand NK Cells[J]. CellMeta. 2016, 24:657–71.
14. 王佳琦,唐茜,朱欣,等.PIM1 过表达对甲状腺乳头状癌细胞代谢组学的影响及意义[J]. 中国普通外科杂志, 2024, 33:11.
15. Wanli Ge,Lingdong Meng,Shuoji Gao,et al. The SIX1/LDHA Axis Promotes Lactate Accumulation and Leads to NK Cell Dysfunction in Pancreatic Cancer[J].Journal of Immunology Research Volume. 2023, 6891636, 21.
16. C Chang, J Qiu, D O'Sullivan, et al. Metabolic competi-tion in the tumor mi-croenvironment is a driver of cancer pro-gression[J]. Cell. 1229–1241, 2015.
17. J Hutcheson, U Balaji, M R, et al. Immunologicand metabolic features of panc-reatic ductal adenocarcinomadefine prognostic subtypes of disease[J]. Clinical Cancer Research. 3606–3617, 2016.
18. An Huang, Haopeng Hong, Jin Gu, et al. Novel hypoxia and lactate metabolis-m related molecular subtyping and prognostic signature for colorectal cancer[J]. Journal of Translational Medicine. 2024, 22:587
19. Jingtong Wu, Pei Hao, Yanyun Fan, et al. Immunological profile of lactylation related genes in Crohn's disease: a comprehensive analysis based on bulk and single cell RNA sequencing data[J]. Journal of Translational Medicine. 2024, 22:300
20. Mingjun Gao,Mengmeng Wang,Wenbo He,et al.Machine learning-based progno--stic model of lactylation-related genes for predicting prognosis and immune in-filtration in patients with lung adenocarcinoma[J].Cancer Cell International. 2024, 24:400.
21. Wang Pei,Zi Lei,Wen Liu,et al. Single-cell transcriptomics analysis reveals that the tumor-infiltrating B cells determine the indolent fate of papillary thyroid ca-rcinoma[J]. Exp Clin Cancer Res. 2025 Mar11;44(1):91.

 doi:10.20944/preprints202603.1110.v1

22. Minjing Zhou,Yufei Shi,Falsh Almohanna,et al. Genome-wide transcriptome anal-ysis and drug target discovery reveal key genes and pathways in thyroid canc--er metastasis[J]. Front Endocrinol (Lausanne). 2025 Feb10:16:1514264.
23. Prakash K,Dhayalan S,Gangadaran P,et al. Natural Killer Cell and Extracellular- Vesicle-Based Immunotherapy in Thyroid Cancer: Advances, Challenges, and Future Perspectives[J]. Cells. 2025 Jul 16;14(14):1087.
24. Xu Lin,Jing Zhang,Gang Xue,et al. APOE Is a Prognostic Biomarker and Corre-lates with Immune Infiltrates in Papillary Thyroid Carcinoma[J]. Cancer. 2022 Mar 14;13(5):1652-1663.
25. Binxin Li,Zhaogen Cai,Lei Chen,et al. Biomarkers associated with papillary thyr-oid carcinoma and Hashimoto's thyroiditis: Bioinformatic analysis and experime-ntal validation[J]. Int Immunopharmacol. 2024 Dec 25;143(Pt 3):113532.
26. Zhiyuan Wang,Xiaoyu Ji,Fan Yang,et al. Interactions between LAMP3+ dendriti-c cells and T-cell subpopulations promote immune evasion in papillary thyroid carcinoma[J]. Immunother Cancer. 2024 May 30;12(5):e008983.
27. Guoli Du,Yefan Yang,Xinxi Li,et al. Th17/Treg cell balance in patients with pa-pillary thyroid carcinoma: a new potential biomarker and therapeutic target[J]. Front Oncol. 2024 Oct 29:14:1325575.
28. Qilan Jiang,Fuyi Wang,Yuan Men,et al. Characteristic expression profile of smal-l nucleolar RNAs and their association with tumor immune microenvironment in papillary thyroid carcinoma[J]. BMC Cancer. 2025 Jul 1;25(1):1073.
29. Zhongfu Pan,Tong Xu,LiSha Li,et al. CREB3L1 promotes tumor growth and m-etastasis of anaplastic thyroid carcinoma by remodeling the tumor microenviron-ment[J]. Melocula Cance. 2022, 21:190.
30. Ramone T,Ghirri A,Matrone A,et al. Molecular Profiling of Low-Risk Papill-ary Thyroid Carcinoma (mPTC) on Active Surveillance[J]. Clin Endocrinol Metab. 2025 Feb 18;110(3):685-692.