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Original Article

Identification of 10 differentially expressed and cuproptosis-related genes in immune infiltration and prognosis of thyroid carcinoma

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Abstract

The association between the cuproptosis-related genes and the immune infiltration and their prognostic value in thyroid carcinoma is still unexplored. Bioinformatics analyses were performed with data obtained from the TCGA dataset. The aberrantly expressed genes were selected. KEGG and GO analyses were conducted to explore the enriched pathways of the up-regulated or down-regulated genes in thyroid carcinoma. Totally 1495 genes were differentially expressed (691 up-regulated, 804 down-regulated) in thyroid carcinoma ($p<0.05$). The 10 cuproptosis-related RNAs (DLD, LIAS, LIPT1, FDX1, DLAT, MTF1, PDHA1, CDKN2A, GLS and PDHB) were also demonstrated to be aberrantly expressed in thyroid carcinoma patients tissues. FDX1 expression was correlated with the overall survival in thyroid carcinoma patients (HR=0.4995, 95% CI: 0.2688-0.9285, p=0.0282). Further multivariate cox regression analysis revealed that DLD (HR=24.8869, 95% CI: 4.48772-138.01181, p=0.00024), and LIAS (HR=7.74092, 95% CI: 1.12194-53.40898, p=0.03783) were associated with the survival of thyroid carcinoma patients. The immune infiltration analysis demonstrated that significant correlation between the 10 cuproptosis-related genes and immune infiltration in thyroid carcinoma ($p<0.01$). We presented the expression profiles of dysregulated genes in thyroid carcinoma. The findings of our study highlighted the potential of cuproptosis-related genes as prognostic biomarkers for thyroid carcinoma.

Keywords: Cuproptosis, Thyroid carcinoma, Prognosis, FDX1, DLD, LIAS.

1. Introduction

Thyroid cancer, which usually arises from follicular or parafollicular C cells, is the ninth most common malignancy, with 586,000 cases worldwide in 2020 [1]. Among the several histological subtypes of thyroid carcinoma, papillary thyroid carcinoma is the most common subtype of thyroid carcinoma and anaplastic thyroid carcinoma is the most fatal subtype with survival rate of six months. Risk factors such as family history and previous head and neck irradiation may contribute to the development of thyroid carcinoma [2]. Currently, effective treatment options for differentiated thyroid cancer (papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC)) are total thyroidectomy, radioactive iodine therapy and thyroid hormone suppressive therapy [3]. In addition, with the growing knowledge of the dynamics of the immune system's interaction with the tumor microenvironment, immunotherapy has emerged as a potential alternative treatment option for advanced thyroid carcinoma [4]. However, the mortality of thyroid carcinoma is elevated during the last past few decades [5]. Therefore, the exploration and identification of genes related to the prognosis and immune infiltration in thyroid carcinoma may contribute to the development of novel anti-cancer strategies.

Copper is a critical trace element significantly involved in the biochemistry of living organisms. Increasing evidence indicates that copper levels show a significant increase in serum and tumor tissues of cancer patients. When copper homeostasis is dysregulated, it may lead to altered intracellular copper levels that may be detrimental to the cells [6]. Cuproptosis is a new type of copper-induced cell death characterized by Tsvetkov et al. [7]. It occurs following disrupted mitochondrial respiration as excess copper binds with the lipid-acylated components of the TCA cycle [7]. Therefore, copper ionophores and copper chelators are suggested to be used in antitumor therapy [8, 9]. A previous study also indicates that copper chelation is an effective therapy that significantly suppresses the BRAF-driven PTC [10]. Furthermore, genes involved in cuproptosis and their association with cancer prognosis should be explored for the targeted therapy of cancers. It has been reported that cuproptosis-related genes are involved in the prognosis of melanoma [11]. Besides, Liuqing Yang et al have pointed that cuproptosis-related genes are biomarkers of the immune microenvironment in head and neck squamous cell carcinoma [12].

In the current study, we investigated the differentially expressed and cuproptosis-related RNAs associated with

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immune infiltration and prognosis of thyroid carcinoma patients. The findings of our study may provide novel therapeutic targets and deepen the underlying mechanism of cuproptosis in thyroid carcinoma.

2. Material and Methods

2.1. Identification of differentially expressed genes

RNAseq data of thyroid carcinoma patients were obtained from the Cancer Genome Atlas (TCGA) database (https://portal.gdc.com). The differentially expressed genes were explored using the R software (Version: 4.0.3), Limma package. Differentially expressed genes were selected under the cutoff value of adjusted $p < 0.05$ and $|\log2FC| > 1$. The boxplot was drawn using the "R" software ggplot2 package; the heatmaps were generated with the "R" software pheatmap package.

2.2. Functional enrichment analysis

The functions of aberrantly expressed RNAs in thyroid carcinoma were explored with the Gene Ontology (GO) enrichment analysis for the molecular function, biological pathways, and cellular components, while the Kyoto Encyclopedia of Genes and Genomes (KEGG) Enrichment Analysis was used to investigate the potential enriched pathways of the up-regulated or down-regulated RNAs. R software with ClusterProfiler package was applied for the corresponding enrichment analyses of the highly expressed or lowly expressed RNAs.

2.3. Prognosis prediction analysis

RNAseq data of RNA levels and patient clinical data were obtained from the TCGA database. Group differences in survival were assessed using log-rank test. R package risk was applied to generate the scatter diagram with high or low FDX1 expression in thyroid carcinoma patients. The ROC analysis was applied to examine the prediction accuracy of FDX1. Log-rank tests and univariate Cox proportional hazards regression were applied for the analysis of p-values and hazard ratio (HR) with 95% confidence interval (CI) in Kaplan-Meier curves [13]. The R packages survival and was used to draw survival prediction curves. *p* value less than 0.05 indicated statistical significance.

2.4. Establishment and assessment of the nomogram

RNAseq data of RNA levels and patient clinical data were obtained from the TCGA dataset. The *p* value, HR and 95% CI of each variable in the forest plot were analyzed by the 'forestplot' R package. The nomogram was constructed using multivariate Cox regression analysis for the prediction of the 1, 2, 3-year overall survival, which presented the potential risk factors for the calculation of survival risks for each patient through the points related to each risk factor using 'rms' R package.

2.5. Tumor-infiltrating immune cell analysis

The correlations among multiple genes: A heatmap of the correlation between 10 cuproptosis-related genes and immune infiltration in thyroid carcinoma was drawn using the "R" software pheatmap package. The correlation coefficients vary with colors and negative correlation was presented as red color while positive correlation was presented as blue color, and the color depth indicated the degree of the correlation. *p* value less than 0.05 was regarded as statistical significance.

2.6. Statistical analysis

The R software (Version: 4.0.3) was applied for statistical analyses in the current work. Wilcox test was applied for the two-group difference comparison. The volcano plot, heatmaps, boxplots, forest plots, ROC curves and calibration plots were drawn by the R software using corresponding packages. Patient survival was analyzed using Kaplan-Meier survival analysis by log-rank test. The univariate and multivariate Cox regression analyses were conducted by the R software for the construction of the nomogram. *p* value less than 0.05 indicated statistical significance.

3. Results

3.1. Differentially expressed RNAs and related signaling pathways in thyroid carcinoma patients

Based on the analysis of RNA-seq data from TCGA cohort, we found 1495 genes differentially expressed (691 highly expressed RNAs and 804 lowly expressed RNAs) in thyroid carcinoma ($|\log2FC| \geq 1$, $p < 0.05$). The volcano plot showed that genes such as LIPH, SLC22A31, RXRG, PRR15, SERPINA1, ZCCHC12, CLDN16, FN1, B3GNT3, SLC34A2, TMPRSS6, DCSTAMP, ARHGAP36 were up-regulated in the tumor tissues of thyroid carcinoma tissues, and genes such as TFF3, CDH16, CCL21, BMP8A, TPO, DIO1 and MT1G were down-regulated in thyroid carcinoma patient tissues (Fig 1A). The

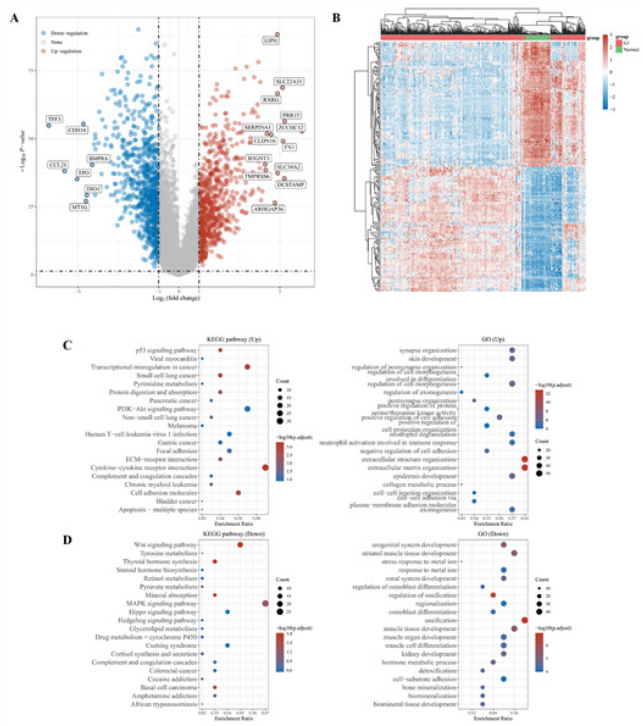

Fig. 1. Differentially expressed genes and KEGG and GO analyses in thyroid carcinoma. (A) The volcano plot showed the differentially expressed genes in tumor tissues compared with normal tissues in thyroid carcinoma patients. Red dots indicate up-regulated genes; blue dots indicate down-regulated genes; grey dots indicate non-significant. (B) The heatmap presented the top 50 up-regulated genes and top 50 down-regulated genes in tumor tissues of thyroid carcinoma patients. KEGG and GO analyses of (C) the up-regulated genes and (D) down-regulated genes in tumor tissues of thyroid carcinoma patients. Colors represent the significance of differential enrichment, the size of the circles represents the number of genes, the larger the circle, the greater the number of genes.

heatmap showed the top 50 highly expressed and lowly expressed RNAs in thyroid carcinoma samples (Fig 1B).

Based on the KEGG and GO analysis, we further investigated the underlying biological functions and related enriched signaling pathways of these aberrantly expressed RNAs. We found that the up-regulated RNAs showed significant enrichment in the p53 signaling pathway in the KEGG pathway analysis, and synapse organization of the GO analysis (Fig 1C), while the down-regulated genes were most significantly enriched in the Wnt signaling pathway in the KEGG pathway analysis and urogenital system development on the GO analysis (Fig 1D). Collectively, differentially expressed RNAs and related signaling pathways were discovered in thyroid carcinoma patients.

3.2. Expression profile of 10 cuproptosis-related RNAs in thyroid carcinoma

We also examined the expression of 10 cuproptosis-related genes in the tumor tissues and normal tissues of thyroid carcinoma patients [14]. The results indicated that DLD, PDHB, LIPT1, PDHA1, FDX1, DLAT, LIAS, MTF1 were significantly down-regulated in the tumor tissues of thyroid carcinoma patients while GLS and CDKN2A exhibited significant up-regulation in the thyroid carcinoma tumor tissues relative to the normal tissue samples (Fig 2A). In a word, 10 cuproptosis-related RNAs were significantly up-or down-regulated in thyroid carcinoma.

The impact of 10 cuproptosis-related RNAs on thyroid carcinoma patient prognosis was explored, and the results indicated that FDX1 acted as a risk factor in thyroid carcinoma patient prognosis (HR=0.4995, 95% CI: 0.2688-0.9285, p=0.0282) (Fig 2B). Among the 10 cuproptosis-related genes, we found that only FDX1 was significantly correlated with the prognosis in thyroid carcinoma patients. High FDX1 expression in thyroid carcinoma patients indicated favorable prognosis, while those with low FDX1 expression had poor prognosis (Fig 2C). In sum, thyroid carcinoma patients with high FDX1 expression had favorable prognoses.

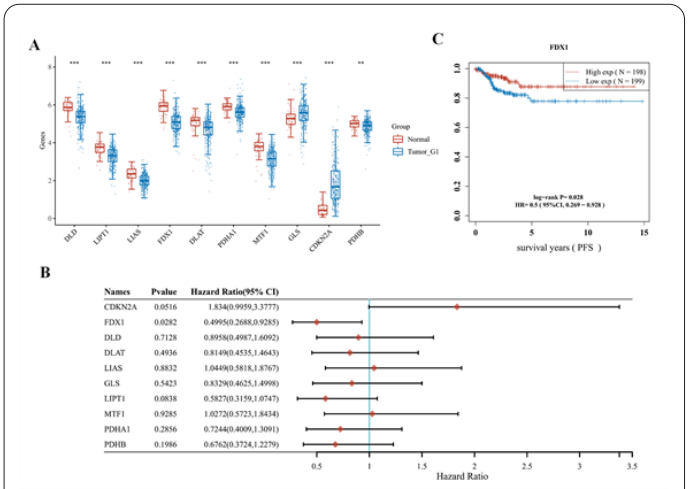

Fig. 2. Expression profile of 10 cuproptosis-related genes in thyroid carcinoma. (A) The expression of 10 cuproptosis-related genes (DLD, LIPT1, LIAS, FDX1, DLAT, PDHA1, MTF1, GLS, CDKN2A, PDHB) in the tumor tissues and normal tissues of thyroid carcinoma patients. (B) The P value, HR and 95% CI of each variable were presented using a forestplot. (C) Kaplan-Meier curves were used to assess the correlation of FDX1 expression and the prognosis in thyroid carcinoma patients.

3.3. Identification of the prognostic value of FDX1 in thyroid carcinoma

The thyroid carcinoma patients were stratified into the high and low FDX1 expression groups, and the scatter plot showed the FDX1 expression of patient samples corresponding to the surviving period and living status of patients. The heatmap of FDX1 levels is presented below. More alive patients were presented in the group with FDX1 high levels (Fig 3A). Then we further investigated the effect of FDX1 on the clinical outcome of thyroid carcinoma patients, and we found that low FDX1 expression predicted poor progression-free survival probability relative to those with high FDX1 expression (HR=0.5, 95% CI: 0.269-0.928, p=0.0282) (Fig 3B). Moreover, the ROC curves were used to analyze the prognostic prediction value of FDX1 in thyroid carcinoma. The results indicated that AUC equals 0.647 for 1-year survival (95% CI: 0.531-0.762), and 0.582 for 3-year survival (95% CI: 0.494-0.67) and 0.599 for 5-year survival (95% CI: 0.497-0.7) (Fig 3C). In a word, FDX1 is a prognosis indicator for thyroid carcinoma patients.

3.4. Identification of independent risk factors in thyroid carcinoma

The p-value, risk coefficient (HR) and confidence interval of 10 cuproptosis-related genes and clinicopathological features in thyroid carcinoma were analyzed via the univariate and multivariate cox regression analyses. The univariate Cox regression analysis suggested that FDX1 (HR=0.49823, 95% CI: 0.2854-0.86976, p=0.01425), was a risk factor in thyroid carcinoma (Fig 4A). According to multivariate cox regression analysis, DLD (HR=24.8869, 95% CI: 4.48772-138.01181, p=0.00024) and LIAS (HR=7.74092, 95% CI: 1.12194-53.40898, p=0.03783) are correlated with overall survival in thyroid carcinoma patients (Fig 4B). Furthermore, a nomogram was genera-

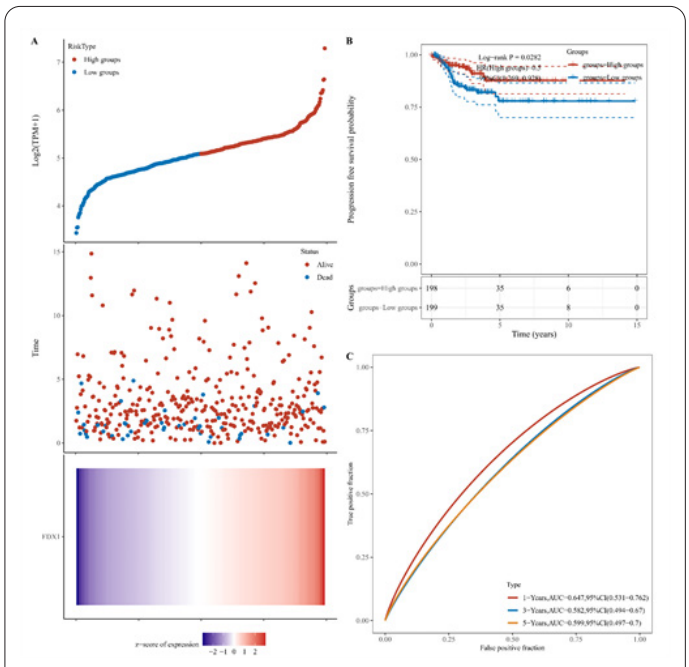

Fig. 3. Identification of the prognostic value of FDX1 in thyroid carcinoma. (A) Distribution of FDX1 expression, survival status of thyroid patients and heatmap of FDX1 expression. (B) Kaplan-Meier analysis was performed to evaluate the survival of patients with high or low FDX1 expression. (C) ROC curves were used to assess the prognostic value of FDX1 in thyroid carcinoma.

ted for the prediction of 1-, 2-, 3-year survival in thyroid carcinoma patients. Risk factors such as DLS, LIAS were incorporated with clinicopathological features including age, pT stage, pM stage. Points were calculated for each of these factors, while the total points of all factors were calculated for the evaluation of survival probability at 1, 2, and 3 years (Fig 4C). Furthermore, Calibration curves for the overall survival nomogram model were presented. The ideal nomogram was shown as the dashed diagonal line, and the 1-, 2- and 3-year of the observed nomogram were presented in indicated colors. The results indicated the agreement between the prediction and observation in the TCGA cohort (Fig 4D). Collectively, FDX1 was a risk factor in thyroid carcinoma and DLD and LIAS are correlated with overall survival and clinicopathological features in thyroid carcinoma patients.

3.5. The correlation of 10 cuproptosis-related RNAs and immune infiltration in thyroid carcinoma

We further analyzed the relationship between immune cell infiltration and 10 cuproptosis-related RNA expression. We found that PDHB was in positive association with macrophage and CD8+ T cell infiltration while was in negative correlation with Myeloid dendritic cell and CD4+ T cell infiltration (**p<0.01). PDHA1 was in positive association with macrophage and CD8+ T cell infiltration while was in negative correlation with the infiltration of CD4+ T cells, neutrophils and Myeloid dendritic cells (**p<0.01). MTF1 was in positive correlation with the immune infiltration of neutrophils, Myeloid dendritic cells, macrophages, B cells and CD8+ T cells while was negatively related to CD4+ T cell infiltration (**p<0.01). LIPT1 was positively associated with the infiltration of macrophages and CD8+ T cells while was negatively related to the infiltration of CD4+ T cells, neutrophils and Myeloid dendritic cells (**p<0.01). LIAS was in positive relation with the infiltration of macrophages and CD8+ T cells, and was negatively associated with the infiltration of neutrophils, CD4+ T cells and Myeloid dendritic cells (**p<0.01). GLS was positively related to the infiltration of Myeloid dendritic cells, macrophages, CD8+ T cells, neutrophils and B cells (**p<0.01). FDX1 expression was in positive relation with the infiltration of CD8+ T cells and macrophages (**p<0.01) while was negatively correlated with the infiltration of neutrophils (*p<0.05), Myeloid dendritic cells (**p<0.01) and CD4+ T cells (**p<0.01). DLD was in positive correlation with infiltration of B cells (**p<0.01), neutrophils (*p<0.05), macrophages (**p<0.01) and CD8+ T cells while was negatively related to infiltration of CD4+ T cells (**p<0.01). DLAT was in positive correlation with infiltration of macrophages, B cells, neutrophils, Myeloid dendritic cells and CD8+ T cells while was negatively associated with CD4+ T cell infiltration (**p<0.01). CDKN2A was positively related to CD4+ T cell, neutrophil and Myeloid dendritic cell immune infiltration while was negatively related to the infiltration of macrophages and CD8+ T cells (**p<0.01) (Fig 5). Collectively, 10 cuproptosis-related RNAs were closely related to immune infiltration in thyroid carcinoma.

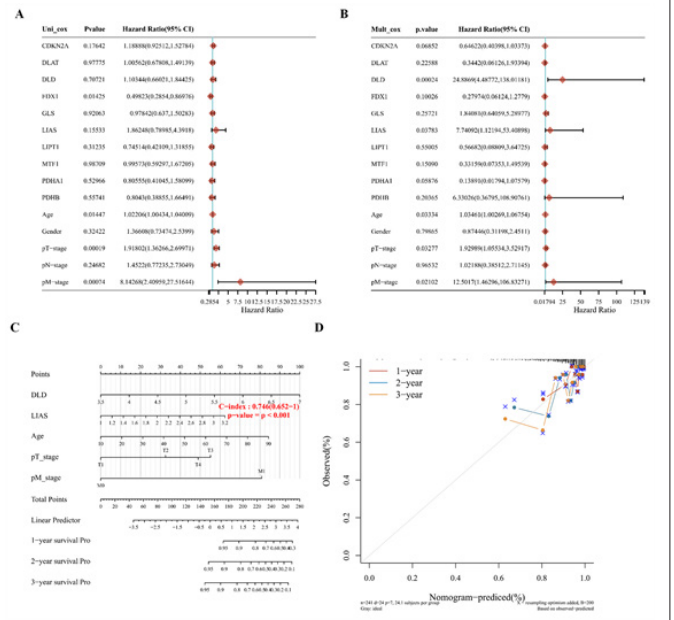

Fig. 4. The correlation of 10 cuproptosis-related genes and clinical outcomes in thyroid carcinoma patients. (A) Univariate and (B) multivariate Cox regression analyses of expression of 10 cuproptosis-related genes and clinicopathological features in thyroid carcinoma patients. (C) The Nomogram was constructed to predict the 1-year, 2-year and 3-year overall survival probability of thyroid carcinoma patients. (D) Calibration curves for the 1-year, 2-year and 3-year survival of the nomogram.

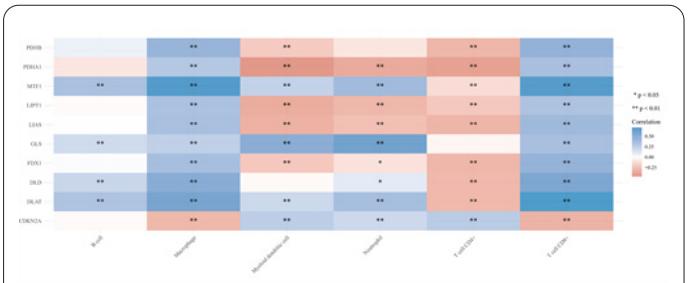

Fig. 5. The correlation of 10 cuproptosis-related genes and immune infiltration in thyroid carcinoma. The correlation between the expression of 10 cuproptosis-related genes (PDHB, PDHA1, MTF1, LIPT1, LIAS, GLS, FDX1, DLD, DLAT, CDKN2A) and immune infiltration of six types of immune cells (B cells, macrophages, Myeloid dendritic cells, neutrophils, CD4+ T cells and CD8+ T cells) in thyroid carcinoma. **p<0.01, *p<0.05.

4. Discussion

In the current work, the differentially expressed RNAs in tumor tissues of thyroid carcinoma patients were screened out. The correlation of 10 cuproptosis-related genes and the immune infiltration and prognosis prediction in thyroid carcinoma was explored and validated with the data of mRNA expression in TCGA database. Our study suggested a potential role of cuproptosis in the prognosis of thyroid carcinoma.

Copper is a commonly used therapeutic metal element in biomedicine, with applications ranging from antimicrobial methods to cancer treatment [15]. Cuproptosis is a non-traditional cell death mechanism associated with protein lipoacylation in the TCA cycle, which may provide new insights into the use of copper toxicity in the treatment of tumors [16]. A large number of literatures have suggested the important role of cuproptosis in cancers [17].

DLD is reported to be involved in various malignancies. Its expression is revealed to be down-regulated in tumor tissues than normal tissues. DLD inhibition blocks lipid

peroxidation and ferrous iron accumulation to suppress ferroptosis in head and neck cancer [18]. LIPT1 is lowly expressed in uterine corpus endometrial carcinoma tumor tissues and acts as a prognostic biomarker in uterine corpus endometrial carcinoma [19]. Previous studies have also revealed the close association between LIAS expression and cancer pathogenesis and immune response [20]. FDX1 and proteoacetylation have been demonstrated to be critically implicated in the regulation of copper-ionophore-induced cell death [7]. FDX1 up-regulation is also reported in hepatocellular carcinoma, and its high levels are correlated with favorable survival [21]. DLAT is a mitochondrial protein implicated in glucose metabolism and is reported to be expressed at high levels in gastric cancer cells and promote cancer cell proliferation by facilitating ATP generation and catabolic reactions [22]. PDHA1 is one of the important components of the pyruvate dehydrogenase complex and is critically involved in glucose metabolism, oxidative phosphorylation as well as tricarboxylic acid cycle in mitochondria [23]. It functions as a prognostic and immune-related biomarker in various malignancies [24]. Previous studies have also demonstrated that miR-21-5p enhances gastric cancer glycolysis and cell proliferation by targeting PDHA1 [25]. MTF1 is a classic metal regulatory transcription factor binding to metal-responsive elements to maintain metal homeostasis [26]. Studies have revealed that MTF1 deficiency suppresses the epithelial to mesenchymal transition as well as cell proliferation in ovarian cancer [27]. GLS plays a key role in the conversion of glutamine to glutamate, which is critically involved in cancer growth. GLS silencing is indicated to exert suppressive effects on osteosarcoma metastasis [28]. CDKN2A is reported with locus on chromosome 9p21 and encodes two anti-cancer proteins $pl6^{INK4A}$ and $p14^{ARF}$ [29]. It functions as a tumor inhibitor and its hypermethylation is suggested as a predictive factor for poor prognosis in colorectal cancer patients [30]. PDHB is an enzyme catalyzing the glucose-derived pyruvate to the acetyl-CoA and is critically implicated in oxidative phosphorylation [31]. A study has found that miR-146b-5p promotes colorectal cancer cell growth, invasion and glycolysis by targeting PDHB [32]. Previous studies have indicated that cuproptosis-related genes can predict the prognosis of tumors [21]. Consistently, our study also found that FDX1 was a risk factor for the clinical outcome of thyroid carcinoma patients and high FDX1 levels were predicted with favorable prognosis in thyroid carcinoma patients. Further multivariate Cox regression analyses revealed that DLD, LIAS and clinical features including age, pT stage, pM stage are were correlated with overall survival in thyroid carcinoma patients.

The immunotherapy has attracted increasing attention in clinical cancer management [33, 34]. The tumor microenvironment (TME) consists of diverse immune cells with anti-cancer or pro-cancer activities. Previous studies have revealed that neutrophils, dendritic cells, mast cells, and macrophages exert an oncogenic effect on thyroid carcinoma while B cells, CD8+ T cells and NK cells play a protective role. Many studies have proved that cuproptosis-related genes are associated with the immune response of tumors [35]. Similarly, in this work, the expression of 10 cuproptosis-related RNAs was significantly correlated with the immune infiltration of immune cells (B cells, macrophages, myeloid dendritic cells, neutrophils, CD4+ T cells and CD8+ T cells) in thyroid carcinoma.

There are some limitations in this study. On the one hand, although prognostic scores focusing on cuproptosis-related RNAs expression performed well in predicting thyroid carcinoma survival, other important genes with predictive value were not considered in this study. On the other hand, given that the predicted features were established and validated by utilizing data from public databases, further biological evidence is required in addition to the statistical evidence we provide.

In conclusion, the association of 10 differentially expressed and cuproptosis-related RNAs with the prognosis and immune infiltration in thyroid carcinoma was identified. The findings of our study may provide novel prognostic and therapeutic biomarkers for thyroid cancer therapy.

Informed consent

The authors report no conflict of interest.

Availability of data and material

We declared that we embedded all data in the manuscript.

Authors' contributions

YW conducted the experiments and wrote the paper; LH, ZY, LM, LW and WL analyzed and organized the data; LD conceived, designed the study and revised the manuscript.

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