Heliyon 9 (2023) e18489

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

MiR-146b-5p regulates the scavenging effect of GPx-3 on peroxide in papillary thyroid cancer cells

Dan Zhang [a,b], Ji-Jun Deng [a], Qin Xu [a], Yang Zeng [c,**], Jun Jiang [a,*]

[a] Department of General Surgery (Thyroid Surgery), The Affiliated Hospital of Southwest Medical University, PR China
[b] Department of Thyroid and Breast Surgery, The Third People's Hospital of Yibin, PR China
[c] Department of Orthodontics, The Affiliated Stomatological Hospital of Southwest Medical University, PR China

ARTICLE INFO

Keywords:
Papillary thyroid carcinoma (PTC)
Glutathione peroxidase 3 (GPx-3)
Oxidative stress (OS)
miR-146b-5p

ABSTRACT

Background: Glutathione peroxidase (GPx) is an important antioxidant enzyme in thyroid follicular cells. Reduced levels of *glutathione peroxidase 3 (GPx-3)* expression in papillary thyroid cancer (PTC) are associated with poor prognosis. However, the reason for the decreased expression level of *GPx-3* in PTC is unclear.

Methods: The expression of *GPx-3* in papillary thyroid carcinoma and adjacent normal tissue (n = 18) was detected by Western blotting. Bioinformatics was used to predict the relationship between the level of GPx-3 and gender, age, lymph node metastasis, stage, $BRAF^{V600E}$ mutation, and recurrence-free survival of patients. The possible upstream microRNAs of GPx-3 were analyzed by bioinformatics tools also. We verified the relationship between GPx-3 and upstream microRNA by dual luciferase reporter assay and enzyme-linked immunosorbent assay (ELISA).

Results: The protein level of GPx-3 decreased in PTC, and analysis of public database datasets suggests that its decreased expression may be associated with the $BRAF^{V600E}$ mutation. MiR-146b-5p was significantly overexpressed in PTC. The dual luciferase reporter assay verified the effect of miR-146b-5p on 3′-UTR of GPx-3 mRNA. Knockdown of miR-146b-5p in thyroid cancer cell lines TPC-1 and BCPAP increased *GPx-3* expression levels, accompanied by an increase in the conversion of glutathione (GSH) to oxidized glutathione (GSSG).

Conclusions: In conclusion, the level of GPx-3 decreases in papillary thyroid carcinoma and impairs intracellular peroxide clearance, due to the inhibitory effect of miR-146b-5p. The accumulation of intracellular peroxides may contribute to the poor prognosis of thyroid cancer.

1. Introduction

Thyroid cancer is the most common malignancy of the endocrine system. The age-standardized incidence rates of thyroid cancer in 2020 were 10.1 per 100,000 women and 3.1 per 100,000 men worldwide. There are regional differences in the incidence of thyroid cancer, ranging from 184 cases per 100,000 women in North America to 17.8 per 100,000 women in East Asia [1]. In general, the prognosis for thyroid cancer is good. The age-standardized mortality rate of thyroid cancer was 0.5 cases/100,000 women and 0.3

* Corresponding author. Department of General Surgery (Thyroid surgery), the Affiliated Hospital of Southwest Medical University, 25 Taiping Street, Jiangyang District, Luzhou 646000, Sichuan province, PR China.
** Corresponding author. Department of Orthodontics, the Affiliated Stomatology Hospital of Southwest Medical University, No.2 Jiangyang Road, Jiangyang District of Luzhou, Sichuan Province, 2646000, China.
E-mail addresses: Zengyang826@swmu.edu.cn (Y. Zeng), jiangjun@swmu.edu.cn (J. Jiang).

https://doi.org/10.1016/j.heliyon.2023.e18489
Received 27 January 2023; Received in revised form 6 July 2023; Accepted 19 July 2023
Available online 20 July 2023
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cases/100,000 men worldwide. In-depth studies on the occurrence and progression of thyroid cancer can help to make individual-based treatments and formulate reasonable and sound public health policies.

Hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS), is required for thyroid hormone synthesis [2]. H_2O_2 is scavenged intracellularly by the enzymatic antioxidant system, including catalase, superoxide dismutase (SOD), GPx-s, peroxiredoxin (PRDX), and the non-enzymatic antioxidation system, including ascorbic acid and glutathione. In tumors, cancer cells often exhibit abnormalities in redox homeostasis. ROS has a dual effect on tumor cells. On the one hand, it can promote the occurrence and development of tumors; on the other hand, it is cytotoxic at high levels [3]. In patients with thyroid cancer, serum levels of oxidants increased while antioxidants decreased [4].

We focus on the expression and function of the GPx-s family in thyroid cancer. The GPx family consists of eight antioxidant enzymes, from GPx-1to GPx-8, which reduce H_2O_2 to H_2O via GSH [5]. GPx-1, GPx-3 and GPx-4 are highly expressed in thyroid follicular cells, with GPx-3 being the most abundant [6]. Silencing the expression of *GPx-3* in papillary thyroid carcinoma can promote tumor metastasis, which may be related to the Wnt/β-catenin signaling pathway [7]. However, the mechanism for the downregulation of *GPx-3* expression in thyroid cancer is unclear. This study aimed to investigate the correlation between low expression of *GPx*-3 and the prognosis of thyroid cancer and the possible mechanism leading to the low expression of *GPx-3*.

2. Method and materials

2.1. Tissues samples

Papillary thyroid carcinoma (PTC) and adjacent normal tissues were collected from 18 patients who underwent lobectomy or total thyroidectomy in the Affiliated Hospital of Southwest Medical University in 2021 (Table 1). Cancerous tissue was taken immediately after thyroidectomy, and adjacent normal tissue was taken from the thyroid gland more than 2 cm away from the cancer. The samples were confirmed by pathologic examination as PTC according to the 2022 WHO Classification of Thyroid Neoplasms [8] and without thyroiditis. Each tissue sample was snap frozen, and stored in a −80 °C refrigerator, and all 18 samples were analyzed by Western blotting.

2.2. Bioinformatics analysis

The GPx-3 mRNA levels and clinically relevant data in thyroid cancer (n = 509) and normal tissues (n = 58) were downloaded from The Cancer Genome Atlas (TCGA) database (https://tcga-data.nci.nih.gov/tcga). Statistical analyses were performed using limma in the Rv3.5.1 environment and the results were visualized using Beeswarm. Data were statistically processed using Kolmogorov Smirnoff, Kruskal, and Wilcox. The Kaplan-Meier plotter (https://kmplot.com/analysis) was used to determine the relationship between the level of GPx-3 and recurrence-free survival (RFS) and the differential expression of GPx-3 between BRAF-mutant PTC and wild-type PTC. Correlations between miRNAs and *GPx-3* expression were analyzed using starBase (https://starbase.sysu.edu.cn/). In the R environment, ggpubr was used to visualize the relationship between GPx-3 mRNA and clinical correlation data. The miRanda,

Table 1
The Clinical characteristics of the 18 PTC patients.

Clinical characteristics	Number
Age	
< 60	15
> = 60	3
Gender	
Male	4
Female	14
Tumor size (cm)	
< 2	10
> = 2	8
Type of surgery	
Lobectomy	4
Total thyroidectomy	14
Multiplicity	
Single	12
Multiple	6
TNM stage[1]	
I	14
II	4
III/IV	0
Recurrence	
Yes	0
No	18

1: Using the American Joint Committee on Cancer (AJCC) Staging for Thyroid Cancer (eighth edition, 2017).

miRmap, PITA and microT algorithms were used to predict hypothetical associations between miRNA and mRNA pairs.

2.3. Western blotting

Tissue samples were crushed with liquid nitrogen and then lysed with Ripa lysis buffer (Beyotime, Shanghai, China) and protease inhibitor (Solarbio, Beijing, China). Total protein concentration was determined using a protein quantification kit (BCA kit) (Beyotime, Shanghai, China). Protein was separated by SDS-PAGE and transferred to the PVDF membrane (Millipore, America). The membranes were incubated with 5% skimmed milk (Beyotime, Shanghai, China) for 2 h at room temperature and then incubated with rabbit anti-human GPx-3 (1:1000, Abcam, UK) at 4 °C overnight. GAPDH (1:5000, Beyotime, Shanghai, China) was used as an internal reference. Finally, the membranes were incubated with the secondary antibody for 2 h at room temperature and visualized using an enhanced chemo luminescence kit (ECL, Beyotime, Shanghai, China).

2.4. Cell line, culture and cell transfection

TPC-1 and BCPAP cell lines (Procell, Wuhan, China) were cultured in RPMI -1640 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Siji Green) and 1% penicillin/streptomycin in a humidified cell culture incubator at 37 °C, 5% CO_2. The miR-146b-5p inhibitor and the inhibitor negative control (NC) were transfected using riboFECT™ CP Transfection Kit (RiboBio, Guangzhou, China) according to the manufacturer's instructions. The final concentration of the transfected miRNA inhibitor and the negative control was 100 nM.

2.5. Enzyme-linked immunosorbent assay (ELISA)

MiR-146b-5p inhibitors and NC inhibitors were transfected into TPC-1 and BCPAP cells, respectively, for 48 h. Cell samples were extracted according to the instructions of the ELISA kit. The expression of *GPx-3* was detected by the human GPx-3 quantitative detection kit (ELISA) (Ruixinbio, Quanzhou, China). GSH and GSSG test kits (Beyotime, Shanghai, China) were used to detect the levels of reduced and oxidized glutathione in the cells.

2.6. Dual-luciferase reporter gene assay

According to NCBI, the 3′ untranslated region (UTR) of *Homo sapiens* GPX3 (NM_001329790) ranges from 773 to 1650 (https://www.ncbi.nlm.nih.gov/gene/2878). We selected a region (130bp) containing its predicted binding site with miR-46b-5p and loaded it into the pmiR-RB-Report vector (Thermo Fisher Scientific). The 5′ restriction enzymes site of the vector is Xhol and the 3′ restriction enzymes site is Notl (Fig. 3b). The forward primer of the PCR was GCTAGCGAGCTCTACACATGGTAGACA and the reverse primer was GCCAGCGGCCGCTTGGACCTAGAGCTG. The length of the PCR amplification product is 250bp, and the sequence is ACACCCTGCTGCTTGCGCCAGCGCCAGGATCAACGTCTAATTCTAGGCGATCGCTCGA-GATGTGTACCATCTGTGTGCCTGCAGCTGTGTAGTGCTGGACAGTGACAACCCTTTCTCTCCAGTTCTCCACTCCAATGATAA-TAGTTCACTTACACCTAAACCCAAAGGAAAAACCAGCTCTAGGTCCAAGCGGCCGCTGGCCGCAATAAAATATCTTTATTTTCATTA-CATCTGTGTGTTGTTTTGGGGA. The underlined part of the sequence is the cloned target sequence, flanked by sequences on the vector backbone. As a control, the corresponding mutated region of GPx-3 (CAGUUCUCC→GUCAAGAG) was also cloned into the pmiR-RB-

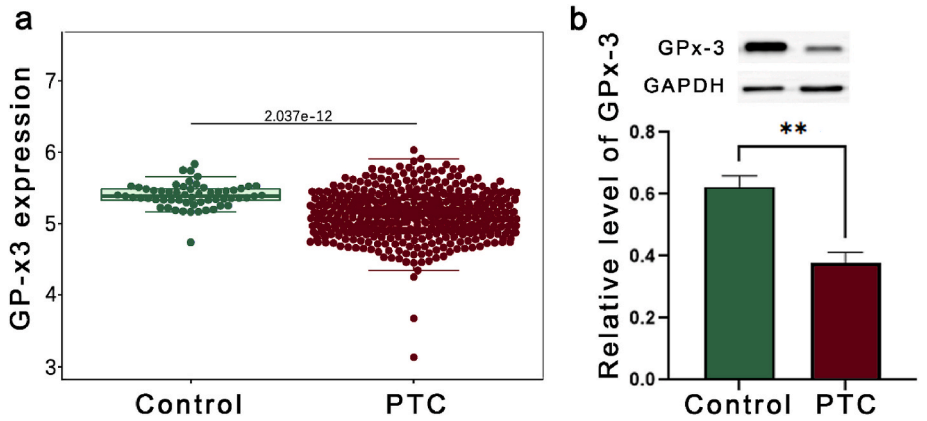

Fig. 1. *GPx-3* was low expressed in papillary thyroid carcinoma. a. The expression of *GPx-3* in papillary thyroid carcinoma was decreased and statistically significant in TCGA (control n = 58, PTC n = 509). b. We collected adjacent normal tissues and papillary thyroid carcinoma tissue samples from patients who underwent lateral lobectomy or total thyroidectomy in the Affiliated Hospital of Southwest Medical University in 2021 (n = 18), here shows the Western blotting image of one of the samples. The protein level of GPx-3 was also decreased in papillary thyroid carcinoma. Data are expressed as mean ± SEM, **p < 0.01.

Report vector. For luciferase assay, 1×10^5/mL HEK293 cells were seeded into LUMITRAC 200 96 well plates (Greiner, Kremsmunster, Austria) and co-transfected with 100 nM miR-146b-5p mimics or miR mimics negative control (NC) using Lipofectamine 3000 for 48 h. Relative renilla/firefly luciferase activity was detected using the Dual-Glo Luciferase Assay System (Promega, Fitchburg, WI, USA) on a GloMax 96 microplate luminometer (Promega) according to the manufacturer's instructions. The relative activity of firefly luciferase in each sample was normalized to renilla luciferase activity.

2.7. Statistical analysis

Results are expressed as the mean ± SEM, and all statistical analyses were performed using GraphPad Prism statistical software. Differences between two groups were compared by unpaired Student's t-test, and multiple groups were compared by one-way ANOVA. $P < 0.05$ was considered statistically significant.

3. Results

3.1. GPx-3 expression was down-regulated in papillary thyroid carcinoma

Using the TCGA database, the expression level of *GPx-3* was investigated in normal thyroid tissue and in thyroid carcinoma (THCA). Compared to normal thyroid tissue, GPx-3 mRNA levels are reduced in THCA (Fig. 1a). In addition, we measured the level of the GPx-3 protein in papillary thyroid carcinoma tissue and adjacent normal tissue. As shown in (Fig. 1b and Supplemental Fig. 1), the protein level of GPx-3 was decreased in PTC tissues.

3.2. The GPx-3 level was associated with age, survival time, distant metastasis, and tumor stage of patients with PTC

The relationship between GPx-3 level and gender, age, tumor stage, lymph node metastasis, and RFS of PTC patients was determined by TCGA thyroid cancer datasets. Patients with low levels of GPx-3 had lower RFS (Fig. 2a). The GPx-3 level was not associated with gender (Fig. 2b). GPx-3 levels in older patients with PTC (age >60 years) are lower than those in younger patients (Fig. 2c). Patients with cervical lymph node metastases (N1) had lower levels of GPx-3 compared to patients without lymph node metastases

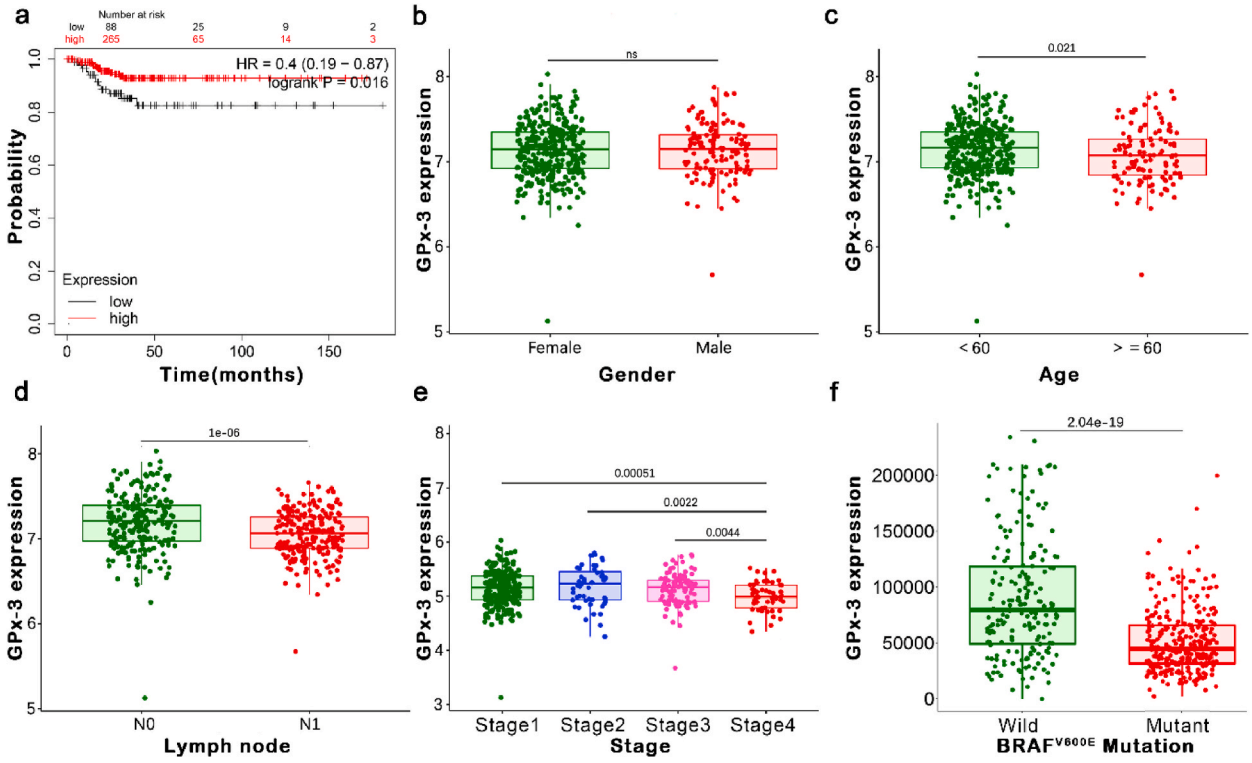

Fig. 2. The level of GPx-3 and clinical characteristics of papillary thyroid carcinoma. a. The level of GPx-3 and survival analysis of papillary thyroid carcinoma. The RFS of papillary thyroid carcinoma with low level of GPx-3 is lower. b. There was no difference in the level of GPx-3 in gender. c. The level of GPx-3 was lower in PTC patients with age ≥ 60 years. d. The level of GPx-3 was lower in PTC patients with cervical lymph node metastasis. e. Comparison of the level of GPx-3 in different stages of papillary thyroid carcinoma. The level of GPx-3 in phase IV PTC was significantly lower than in phases I, II and III. f. The level of GPx-3 was lower in PTC with $BRAF^{V600E}$ mutation.

(N0) (Fig. 2d). Lower levels of GPx-3 were associated with higher malignancy of thyroid cancer, as the level of GPx-3 was significantly lower in stage IV thyroid cancer than in stages I, II, and III (Fig. 2e). Thyroid cancers with $BRAF^{V600E}$ mutations had lower GPx-3 levels (Fig. 2f).

3.3. MiR-146b-5p regulated GPx-3 levels

The reason for the decrease in *GPx-3* expression was not clear. We predicted miRNAs that might regulate *GPx-3* expression levels using miRanda, miRmap, PITA and microT algorithms (Table 2) and excluded the miRNAs at low levels in thyroid tissue. We found that miR-146b-5p and miR-34a-5p were upregulated in thyroid cancer [9,10]. The correlation between the high level of miR-146b-5p and miR-34a-5p and the low level of GPx-3 in PTC was evaluated. There was a weak correlation between miR-34a-5p and the GPx-3 level ($r = -0.088$) (Supplementary Fig. 2), while miR-146b-5p was moderately correlated with the GPx-3 level ($r = -0.447$) (Fig. 3a). Furthermore, miR-146b-5p could affect the 3′UTR of GPx-3 as confirmed by a dual-luciferase reporter assay (Fig. 3b).

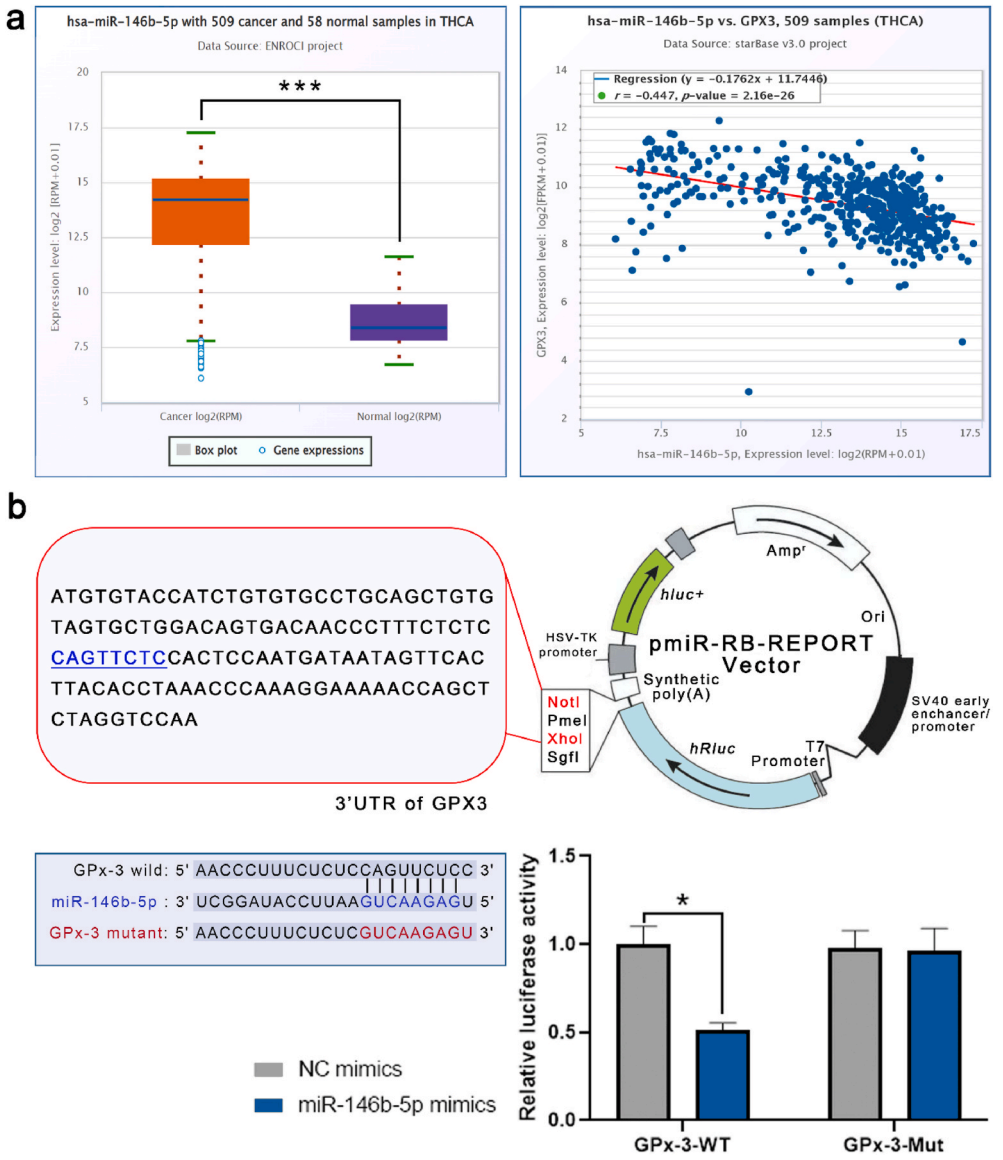

Fig. 3. MiR-146b-5p may regulate the expression of *GPx-3* in PTC. a. In thyroid cancer, there was a negative correlation between the levels of GPx-3 and miR-146b-5p ($r = -0.447$). b. The 3′UTR of GPx-3 containing the binding site (highlighted by underline) for miR-146b-5p was cloned into pmiR-RN-Report between the NotI and XhoI restriction sites. The simulated sequence shows the possible binding sites between the 3′-UTR of GPx-3 and miR-146b-5p. Dual-luciferase reporter assay verified the binding sequence between the molecules. *$p < 0.05$, * * *$p < 0.001$.

3.4. MiR-146b-5p negatively regulates GPx-3 expression in thyroid cancer cell lines

When the miR-146b-5p inhibitor was transfected into thyroid cancer cell lines TPC-1 and BCPAP, respectively, for 48h, the expression level of *GPx-3* was upregulated (Fig. 4a and b). GPx-s catalyzes the glutathione (GSH) to oxidized glutathione (GSSG) and decomposes H_2O_2 to H_2O. After miR-146b-5p knockdown, the GSSG/GSH ratio increased, indicating the increased activity of GPx-3 (Fig. 4a and b).

4. Discussion

Iodination of thyroglobulin requires a high concentration of hydrogen peroxide (H_2O_2), which is present in thyroid follicular cells. However, excessive level of H_2O_2 can damage organelles including DNA [12–15] and cause thyroid tumors or autoimmune thyroid disease [16,17]. Under normal circumstances, follicular thyroid cells have a robust anti-peroxidase system in that includes the GPx-s family. GPx-s uses GSH as a reducing agent to degrade H2O2 and produce GSSH and H_2O. In thyroid cancer, the balance between the peroxide and antioxidant enzyme systems is disturbed, resulting in increased levels of reactive oxygen species [18,19] and decreased activity of the antioxidant enzyme system [4,20,21]. Low expression of *GPx-3* in thyroid cancer has been shown to be associated with cancer metastasis and poor prognosis [7,22]. The dataset from TCGA showed that low expression of *GPx-3* was correlated with lymph node metastasis, older age, advanced stages, low recurrence-free survival (RFS), and $BRAF^{V600E}$ mutation (Figs. 1 and 2). However, the mechanism by which GPx-3 levels are reduced in thyroid cancer is unclear.

MiR-146b-5p is highly expressed in papillary thyroid carcinoma, especially when associated with by $BRAF^{V600E}$ mutation [11]. Its high expression correlates with lymph node metastasis, extrathyroidal invasion, and clinical stage of thyroid cancer [16,23–27]. Interestingly, a study showed that miR-146 significantly decreased the expression of catalase [16]. In the present study, bioinformatics tools predicted that miR-146b-5p could suppress GPx-3 protein level and that the increase of miR-146b-5p might be related to the low expression of *GPx-3*. Dual-luciferase reporter assays confirmed the effect of miR-146b-5p on the 3′UTR of GPx-3 mRNA (Fig. 3b). The transfection experiment with miR-146b-5p inhibitor showed that downregulation of miR-146b-5p could increase the expression level of *GPx-3* and increase the conversion rate of GSSG/GSH, which is an important index to evaluate the activity of GPx-s (Fig. 4 a and b). These results suggest that the increase of miR-146b-5p plays an important role in the decrease of GPx-3 during the occurrence of thyroid cancer, which in turn impairs the redox balance of thyroid cancer cells.

Abnormal expression of the GPx-s family has been associated with various cancers [28–34]. In this study, we investigated why GPx-3 is decreased in thyroid cancer. miR-146b-5p is a key gene expression regulator in thyroid cancer. Under its targeted inhibition, the level of GPx-3 decreases and could lead to the accumulation of intracellular superoxide and tumor progression (Fig. 4c). Restoration of GPx-3 level by blocking miR-146b-5p may improve the prognosis of thyroid cancer patients.

5. Conclusion

The Decreased GPx-3 levels in papillary thyroid carcinoma is partially due to miR-146b-5p overexpression that targets GPx-3 mRNA. The reduction of GPx-3 impairs the clearance of intracellular superoxide, and the accumulation of superoxide may lead to poor prognosis in thyroid cancer. Indeed, we observed that low GPx-3 levels were associated with lymph node metastasis, older age, advanced stages, $BRAF^{V600E}$ mutation, and poorer recurrence-free survival in thyroid cancer patients.

Declarations

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee

Table 2
MiRNAs that may target *GPx3*.

miRNAid	miRNAname	geneID	chromosome	strand	clipExpNum	RBP	merClass
MIMAT0000421	hsa-miR-122-5p	ENSG00000211445	chr5	+	3	AGO1-4,AGO2	7mer-1A
MIMAT0000690	hsa-miR-296-5p	ENSG00000211445	chr5	+	3	AGO1-4,AGO2	8mer
MIMAT0000733	hsa-miR-379-5p	ENSG00000211445	chr5	+	3	AGO1-4,AGO2	8mer
MIMAT0000255	hsa-miR-34a-5p	ENSG00000211445	chr5	+	2	AGO1-4	7mer-m8
MIMAT0000421	hsa-miR-122-5p	ENSG00000211445	chr5	+	2	AGO1-4	7mer-1A
MIMAT0000449	hsa-miR-146a-5p	ENSG00000211445	chr5	+	2	AGO1-4	7mer-m8
MIMAT0000455	hsa-miR-185-5p	ENSG00000211445	chr5	+	2	AGO1-4	8mer
MIMAT0000686	hsa-miR-34c-5p	ENSG00000211445	chr5	+	2	AGO1-4	7mer-m8
MIMAT0001541	hsa-miR-449a	ENSG00000211445	chr5	+	2	AGO1-4	7mer-m8
MIMAT0002809	hsa-miR-146b-5p	ENSG00000211445	chr5	+	2	AGO1-4	7mer-m8
MIMAT0003327	hsa-miR-449b-5p	ENSG00000211445	chr5	+	2	AGO1-4	7mer-m8

The miRanda, miRmap, PITA and mictoT algorithms were used to predict hypothetical associations between microRNA and GPx-3 mRNA. In this table, miRNAs with high prediction scores for binding to the 3′-UTR of GPx-3 are listed. (ClipExpNum, the number of supporting Ago CLIP-seq experiments).

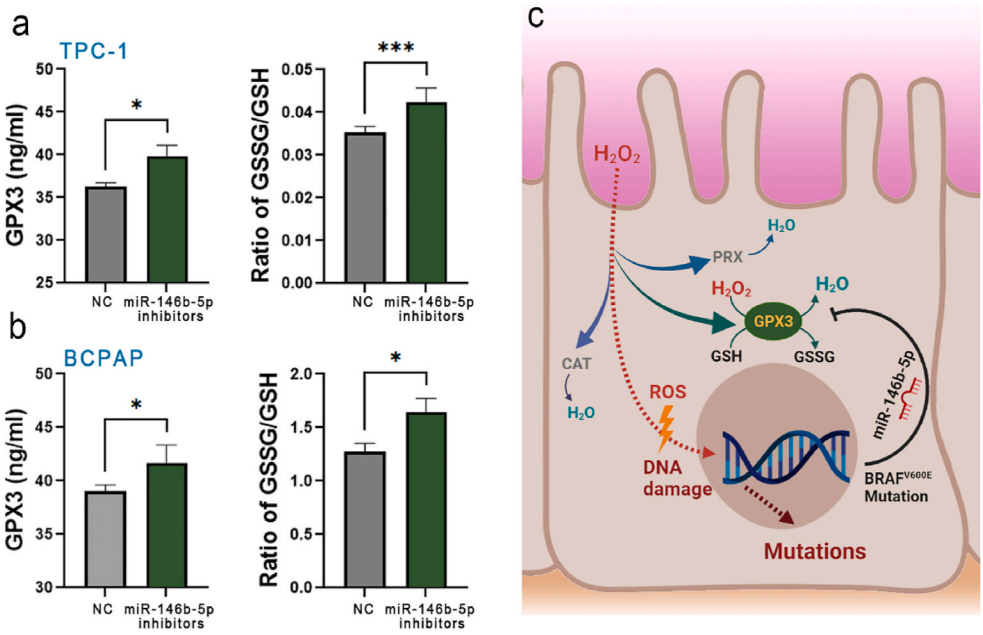

Fig. 4. miR-146b-5p regulated the expression of *GPx-3* in PTC and inhibited the transformation of GSH to GSSG. a. Knockdown of miR-146b-5p in TPC-1 cells increased the protein level of GPx-3 and upregulated the ratio of GSSG/GSH. b. The same result was observed in BCPAP cells. c. MiR-146b-5p affects the mechanism of peroxide scavenging in papillary thyroid carcinoma cells by regulating the level of GPx-3 protein. H_2O_2 is necessary for thyroid cells to synthesize thyroid hormone, and there is a high concentration of H_2O_2 in thyroid follicle cells. Normally, thyroid follicle cells eliminate excess hydrogen peroxide through the CAT, PRDX, and GPx-s systems to prevent H_2O_2 damage to the nuclear genome. In papillary thyroid carcinoma cells, the level of miR-146b-5p was significantly increased due to the $BRAF^{V600E}$ mutation [11]. MiR-146b-5p affects H_2O_2 clearance by directly targeting GPx-3 (its efficiency can be evaluated by the GSSG/GSH ratio). The accumulation of excessive peroxides in cells may cause continuous DNA damage and induce further mutations, thus affecting the prognosis of PTC. *$p < 0.05$, ***$p < 0.001$.

of the Affiliated Hospital of Southwest Medical University (Number: 20210110-008).

Author contribution statement

Dan Zhang: Conceived and designed the experiments; Performed the experiments; Wrote the paper.
Ji-Jun Deng; Qin Xu: Performed the experiments.
Jun Jiang: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.
Yang Zeng: Analyzed and interpreted the data.

Data availability statement

Data included in article/supp. material/referenced in article.

Funding statement

This study is supported by National Natural Science Foundation of China (N82070288), Science & Technology Department of Sichuan Province (2022YFS0627), Health Commission of Sichuan Province (21PJ100) and Office of Science and Technology and Intellectual Property of Luzhou (2022-JYJ-131).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper..

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e18489.

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