Clinical implications of TPO and AOX1 in pediatric papillary thyroid carcinoma

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Background: Thyroid carcinoma is a common pediatric head and neck cancer, of which papillary thyroid cancer (PTC) is the most common type. Previously, we found that thyroid peroxidase (*TPO*) and aldehyde oxidase 1 (*AOX1*) were differentially expressed in PTC. This study explored the clinical importance of *TPO* and *AOX1* in the diagnosis and prognosis of PTC in children.

Methods: Both *TPO* and *AOX1* expression in PTC were analyzed using datasets from Gene Expression Omnibus (GEO). TPO and AOX1 protein levels in plasma from patients with PTC and non-tumor controls were detected via enzyme-linked immunosorbent assay (ELISA). The diagnostic accuracy of TPO and AOX1 was assessed using receiver operating characteristic (ROC) curve analysis. The association between gene expression levels and patient survival was explored using the Kaplan-Meier plotter online database.

Results: The results revealed that *TPO* and *AOX1* expression was significantly downregulated in four independent datasets (GSE33630, GSE27155, GSE3678, and GSE3467). TPO and AOX1 protein levels in blood plasma were significantly decreased in patients with PTC. Quantitative analysis demonstrated that TPO and AOX1 levels in plasma had satisfactory predictive performance and the ability to discriminate PTC from healthy samples. Prognostic analysis demonstrated that low levels of *TPO* and *AOX1* were markedly associated with poor survival in patients with PTC.

Conclusions: In summary, these results implied that TPO and AOX1 could serve as novel biomarkers for the diagnosis and prognosis of pediatric PTC.

Keywords: Pediatrics; papillary thyroid carcinoma; thyroid peroxidase; aldehyde oxidase

Introduction

Global cancer statistics revealed that 567,000 cases of thyroid cancer occur globally each year, ranking ninth among malignancies (1). Papillary thyroid cancer (PTC) is the most common type of thyroid cancer, accounting for at least 90% of all childhood cases. The incidence of PTC is increasing in many countries, including the USA, the UK, Denmark, and China (2-5), and the incidence rates increased more rapidly from 2006 to 2013 than from 1973 to 2006 (6). Because the 5-year survival of PTC usually exceeds 95% (7), it is considered a "curable" cancer. Occasionally, this malignancy can dedifferentiate into aggressive thyroid cancers, and more than 25% of patients with PTC experience recurrence during long-term follow-up (8). It is well known that the *BRAF (V600E)* mutation, *RAS* mutation, and *RET/PTC* rearrangement are common gene alterations in PTC (9). In addition to gene mutations, RNA biomarkers and proteome-based biomarkers were recently identified, and it is hoped that these biomarkers can discriminate this cancer from non-cancer (8). However, the etiology and molecular biology of PTC remain clear. Previously, we found that both thyroid peroxidase (*TPO*) and aldehyde oxidase 1 (*AOX1*) were downregulated in PTC (10,11). However, further experimental verification and studies of the clinical importance of *TPO* and *AOX1* in PTC must be conducted. Careful assessment of genetic features is considered useful for predicting clinical outcomes and planning treatment.

As a key enzyme involved in thyroid hormone synthesis, TPO is essential for thyroid function. Previously, peroxidase activity was detected at lower levels in thyroid carcinoma than in normal thyroid glands (12). *TPO* gene mutation and expression changes are also involved in thyroid cancer. A case-control study reported that a *TPO* variant is associated with thyroid carcinoma (13). Our previous study and other integrated analyses demonstrated that *TPO* was significantly downregulated in PTC (10,14). Pathologically, immunohistochemical staining verified that TPO can distinguish PTC from benign thyroid lesions (15). However, TPO protein levels in blood, which might be a promising non-invasive index, have not been explored to date.

AOX1 is well described as a xenobiotic-metabolizing enzyme that plays roles in the metabolism of nitrogenous heterocyclic compounds and regulation of reactive oxygen species (ROS) and lipid metabolism (16,17). A proteomic analysis found that *AOX1* expression was high in normal tissues but completely lacking in hepatocellular and pancreatic cancer (16,18). Moreover, reduced *AOX1* expression is correlated with the hepatocellular tumor stage and metastatic/regional lymph node states (16). However, the association between *AOX1* expression and PTC pathogenesis is unclear.

Therefore, we measured the protein content of TPO and AOX1 in plasma from children with PTC. Diagnostic analysis was performed to determine whether TPO and AOX1 in plasma are promising biomarkers for PTC diagnosis. Using an online database, survival analysis was conducted to evaluate their clinical importance for PTC prognosis.

We present the following article in accordance with the REMARK reporting checklist (available at http://dx.doi.org/10.21037/tp-20-301).

Methods

Blood samples and clinical information

This study follows the Helsinki Declaration (as revised in 2013) and was approved by the Ethics Committee in Beijing Children's Hospital, Capital Medical University (2020-k-168). In total, data for 64 patients diagnosed with PTC (February 2012 to May 2019) were collected from the medical record system, and 40 blood samples were available in our Biobank of Clinical Resources. Forty cancer-free blood samples (physical examination) matched for age and gender served as the control group. The TNM pretreatment staging classification was used for the clinical staging of PTC. Informed consent was provided by each patient and/or his/her legal guardians before samples were collected and stored in the biobank.

Public data preprocessing

To verify the expression of *TPO* and *AOX1*, several Gene Expression Omnibus (GEO) datasets were analyzed. Series matrix files of GSE33630 (49 PTC, 45 normal), GSE27155 (51 PTC, 4 normal), GSE3678 (7 PTC, 7 normal), and GSE3467 (9 PTC, 9 normal) were downloaded. Normalized values of both *TPO* and *AOX1* were then selected to verify their expression in PTC and normal thyroid tissue.

TPO and AOX1 detection

According to the standard operating procedure in the biobank, venous blood samples were taken from each patient

at the time of diagnosis. Blood samples were collected in EDTA anticoagulant and centrifuged at 3,000 rpm for 5 min, and the supernatant was stored frozen at –80 ℃ until analysis. The concentrations of TPO and AOX1 in plasma were measured using enzyme-linked immunosorbent assay (ELISA) kits (TPO, Cusabio, USA; AOX1, Abbexa, UK) according to the manufacturer's instructions.

Diagnostic testing

Receiver operating characteristic (ROC) curves were used to evaluate classifiers in clinical diagnosis. To assess the predictive accuracy of TPO and AOX1 levels in pediatric PTC, ROC curves were generated using the TPO and AOX1 concentrations in plasma from pediatric patients and cancer-free controls. The area under the ROC curve (AUC) was calculated and used to evaluate the diagnostic usefulness of TPO and AOX1.

Prognostic analysis

The prognostic importance of *TPO* and *AOX1* expression was evaluated using the Kaplan–Meier plotter (www.kmplot.com), a widely used online database for survival analysis (19). In the database, gene expression data and relapse-free survival (RFS) information were downloaded from GEO, the European Genome-phenome Archive, and The Cancer Genome Atlas. This database is capable of assessing the effects of 54,000 genes (mRNA, miRNA, protein) on survival in 21 cancer types including PTC. The database contains 502 cases of PTC. Briefly, *TPO* and *AOX1* were entered in the database to acquire RFS plots. Hazard ratios with 95% confidence intervals and log-rank p-values were calculated and displayed on the main plots.

Statistical analysis

All data and graphs were generated using GraphPad Prism 7.0. An unpaired *t*-test was performed to analyze differences between two groups. P<0.05 indicated statistical significance. All results were expressed as the mean ± SD.

Results

Clinical characteristics of patients

A summary of the clinicopathological characteristics of the patients, including age at diagnosis, gender, and TNM

Table 1 Clinical characteristics of pediatric PTC patients investigated

Parameters	Classes	Number of patients (%)
Age of diagnosis	Total	64
	Mean age	9.49 (3.25–15.95)
	0–6	11 (17.1)
	6–10	23 (36.0)
	>10	30 (46.9)
Gender	Male	20 (31.3)
	Female	44 (68.7)
T stage	T1	9 (14.1)
	T2	18 (28.1)
	T3	21 (32.8)
	T4	16 (25)
Lymph node status	Positive	52 (81.2)
	Negative	12 (18.8)
TNM Stage	I	51 (79.6)
	II	13 (20.4)

PTC, papillary thyroid carcinoma; TNM, tumor staging system based on primary tumor (T), regional lymph nodes (N) and distant metastasis (M).

stage, is presented in *Table 1*. The patients ranged in age from 4.02 to 15.33 years (mean, 10.06 years), and 46.9% of the patients were ≥10 years old. The patients included 20 boys (31.3%) and 44 girls (68.7%), giving a male/female ratio of 1:2.2. The numbers of lesions classified as T1, T2, T3, and T4 were 9 (14.1%), 18 (28.1%), 21 (32.8%), and 16 (25%), respectively. Regarding regional lymph nodes metastasis, 52 cases (81.2%) were positive, and 12 cases (18.8%) were negative. Fifty-one patients (79.6%) were diagnosed with TNM stage I disease, and 13 patients (20.4%) were diagnosed with stage II disease.

Gene expression of TPO and AOX1

As presented in the GSE33630, GSE27155, and GSE3678 datasets, both *TPO* and *AOX1* were expressed at significantly lower level in PTC tumors (*Figure 1*). Additionally, *TPO* and *AOX1* expression was also lower in tumor tissues than in paired normal thyroid tissues (GSE3467, *Figure 1D*). Although *TPO* is expressed exclusively in the thyroid, AOX1 concentrations in plasma might be influenced
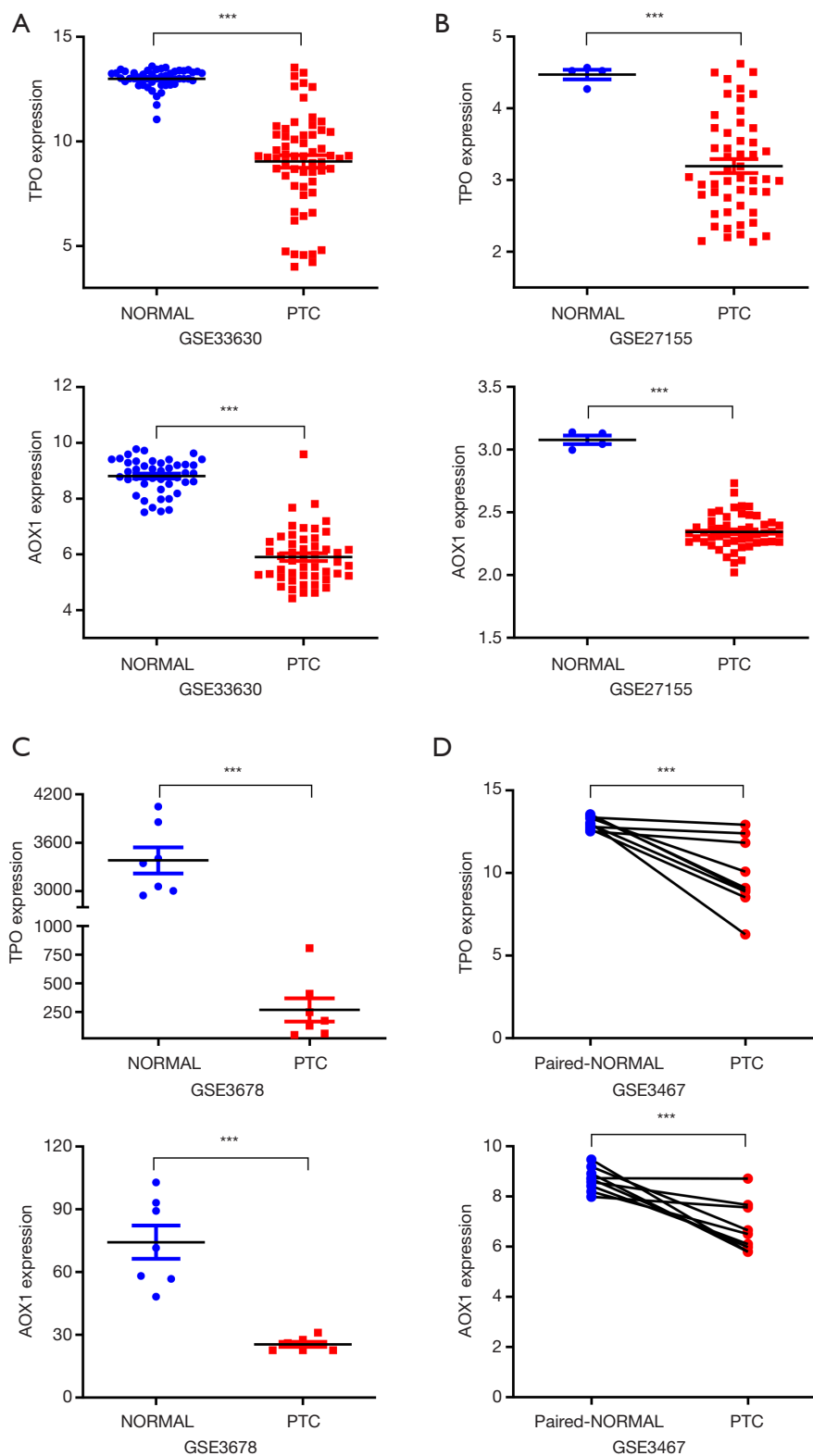
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Figure 1 The expression of TPO and AOX1 in different GEO datasets. (A) GSE33630, (B) GSE27155, (C) GSE3678 and (D) GSE3467. Both TPO and AOX1 were significantly decreased in PTC compared with those in normal thyroid. ***, P<0.001.
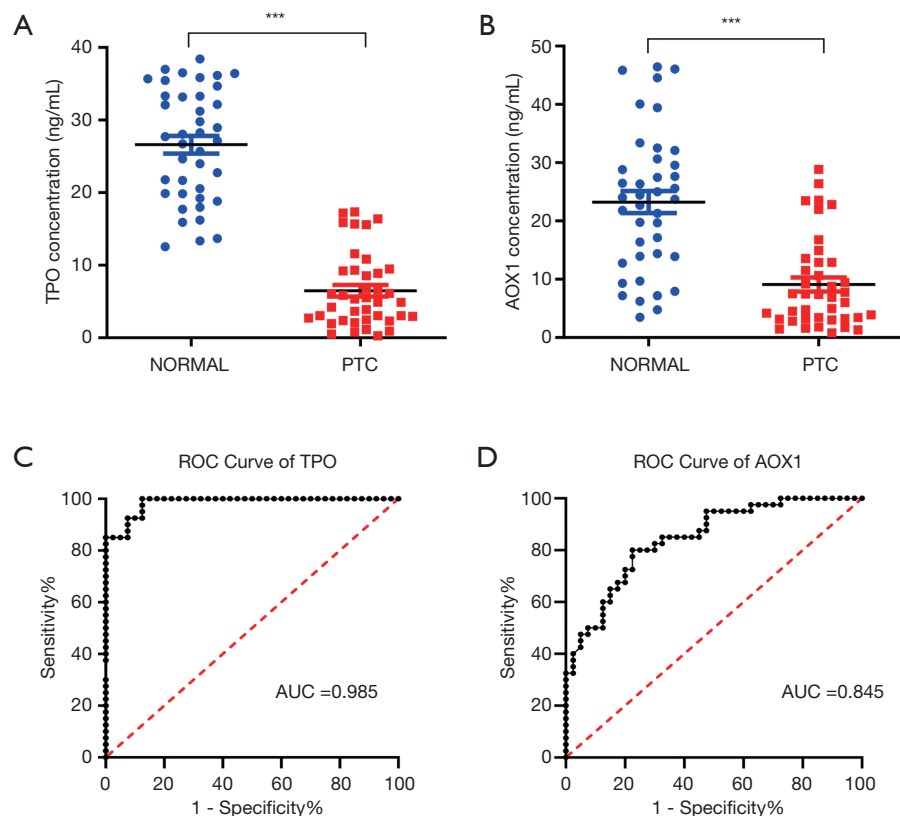
Figure 2 TPO and AOX1 protein content in plasma and ROC curves. (A) TPO protein content and (B) AOX1 content in PTC and cancer-free samples. (C) ROC curve for TPO (AUC =0.985). (D) ROC curve for AOX1 (AUC =0.845). ***, P<0.001.

by other organs or systems. ELISA was further used to explore TPO and AOX1 protein concentrations in plasma. The results illustrated that their plasma levels were significantly lower in children with PTC than in cancer-free controls (*Figure 2A,B*). These independent datasets and experimental validation confirmed that *TPO* and *AOX1* expression was downregulated at both the gene and protein levels in PTC.

Diagnostic effects of TPO and AOX1

To assess the predictive accuracy of TPO and AOX1 in plasma, ROC analysis was performed, and AUC was calculated using protein concentrations. The results indicated that TPO had an AUC larger than 0.98 (*Figure 2C*), and AOX1 had an AUC of 0.845 (*Figure 2D*). Our ROC analysis indicated that TPO and AOX1 contents in plasma had excellent predictive performance for discriminating PTC from healthy samples.

Prognostic implication of TPO and AOX1

We further determined the prognostic value of *TPO* and *AOX1*, and RFS curves were plotted on www.kmplot.com for patients with PTC (n=502). As presented in *Figure 3*, patients with low *TPO* and *AOX1* expression had poorer RFS rates than those with high expression (P=0.002 and P=0.038, respectively). These results suggested that *TPO* and *AOX1* are potential prognostic markers for PTC.

Discussion

The incidence of pediatric PTC is increasing. To explore its pathogenesis, high-throughput studies have been performed in genetic, epigenetic, and proteomic fields (14,20,21). Various alterations of genes, non-coding RNAs, and proteins have been identified, but it is necessary to verify the reliability of all screened targets in clinical specimens (22,23). In this study, we confirmed that the expression of both *TPO* and *AOX1* was downregulated in PTC tissue,
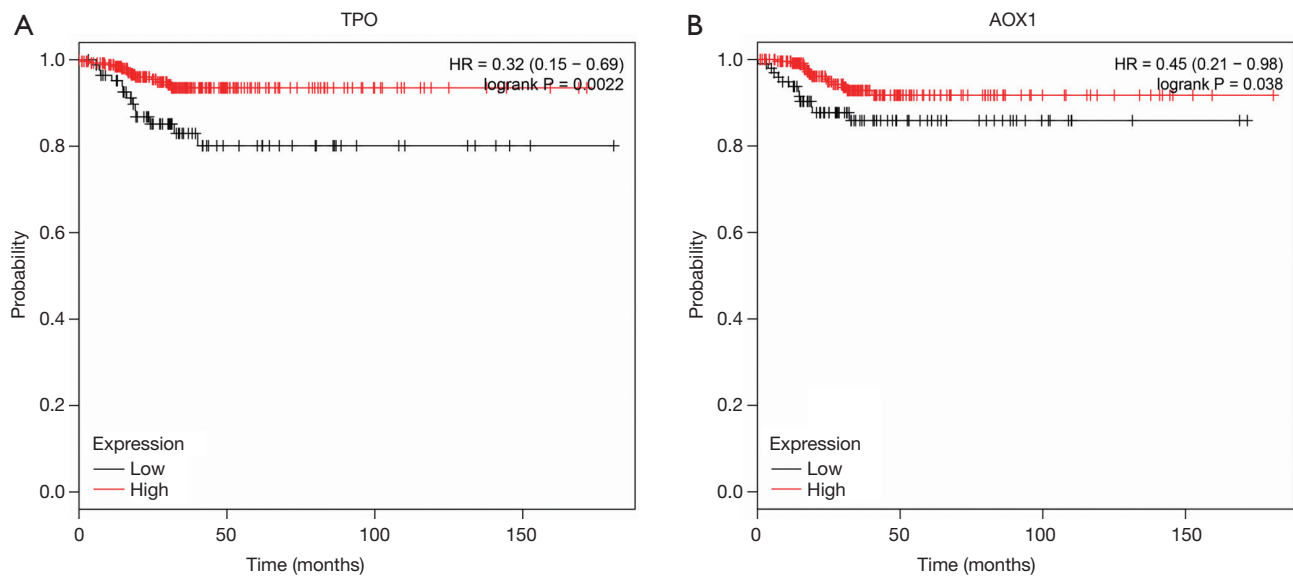
Figure 3 Kaplan-Meier relapse-free survival (RFS) analysis of TPO and AOX1 expression. (A) The survival curves for TPO and (B) AOX1 in low-expression versus high-expression patients.

and their contents in plasma from patients with PTC were decreased. We clarified that TPO and AOX1 might be promising biomarkers for the diagnosis and prognosis of pediatric PTC.

Evidence has illustrated that the incidence of PTC increases with age, and the incidence in adolescents is 10-fold higher than that of younger children (24). In our study, 82.9% of cases of PTC occurred in children >6 years old. In accordance with previous literature (25), our study demonstrated that females have a higher incidence, with a female/male ratio of 2.2:1, suggesting a female preponderance in PTC. The TNM classification system is the most widely used cancer staging system for most types of cancer. Pediatric patients are classified into stage II if distant metastases are identified; otherwise, all pediatric patients are considered to have stage I disease (26). Compared with the findings in adults, children had larger primary tumors and more lymph node metastases (LNMs) or distant metastases in prior analyses (25,27). In this study, more patients had LNM (81.2%), and 20.4% of patients had distant metastasis, indicating that pediatric PTC is more grossly invasive.

TPO expression was significantly downregulated in four independent datasets (GSE33630, GSE27155, GSE3678, and GSE3467), and its protein contents in plasma from patients with PTC were also significantly decreased. Previous research reported that *TPO* expression

was downregulated in PTC tumor tissue (14,28), which is consistent with our results. TPO protein content in plasma was detected for the first time in this study. TPO is an enzyme involved in thyroid hormone synthesis and iodine metabolism that is essential for thyroid function. For thyroid disorders, TPO autoantibody has been detected in 88% of all patients with various thyroid and autoimmune diseases (29). In thyroid cancer, the expression of *TPO* is lower in BRAF mutation-positive tumors (30). It is well known that the *BRAF (V600E)* mutation and *RET/PTC* rearrangement are common gene alterations in PTC (9). A previous study demonstrated that the *BRAF (V600E)* mutation, but not *RET/PTC* rearrangement, is correlated with lower *TPO* expression (31). In terms of mechanism, *TPO* downregulation is reportedly driven by constitutive hyperactivation of the MAP kinase signaling pathway mediated by oncogenic *BRAF (V600E)* (31-33). As a result, *BRAF* genetic alterations may contribute to the regulation of *TPO* expression.

AOX1 is a metabolizing enzyme with poorly understood physiological function and substrates. *AOX1* has critical roles in xenobiotic metabolism and the regulation of ROS generation (16). In the present study, *AOX1* expression in PTC tissue and its content in plasma were significantly reduced. A previous proteomic analysis found that *AOX1* was deregulated in pancreatic ductal adenocarcinoma (PDAC), and immunohistochemical analysis verified that

AOX1 was strongly expressed in approximately 70% of histologically normal pancreatic cells but absent in PDAC cells (18). Another study demonstrated that reduced *AOX1* expression was correlated with tumor stage and metastasis in hepatocellular carcinoma (16). Meanwhile, the function of *AOX1* remains controversial in different tumors because *AOX1* was reported to promote the proliferation of prostate cancer and colorectal cancer but to inhibit the development of breast cancer (34-36). Mechanically, a molecular study demonstrated that nuclear factor erythroid 2-related factor 2 (Nrf2) binds to *AOX1* and strongly activates *AOX1* expression (37). Because Kelch-like ECH-associated protein 1 (Keap1)/Nrf2 signaling is a key mediator in thyroid pathophysiology and thyroid cancer, the regulation of *AOX1* by the Keap/Nrf2 pathway might account for part of the pathogenesis of PTC (38).

Both gene expression in tissues and protein contents in plasma have promising diagnostic and prognostic potential in many tumors (39-41). A previously study reported that serum TPO was not a useful marker for patients with differentiated thyroid carcinoma (42). However, our ROC analysis indicated that the calculated AUCs for TPO and AOX1 were 0.98 and 0.845, respectively. Because TPO can improve the diagnosis accuracy in discriminating well-differentiated thyroid carcinomas from benign tumors, it might be a useful differential diagnosis marker of in PTC (43,44). Moreover, a gene panel containing *TPO* had good sensitivity (91%) and specificity (81%) in differentiating PTC from normal thyroid tissue (45). These results revealed that TPO and AOX1 have excellent predictive performance, thus verifying their potential use for clinical diagnosis.

To further explore the prognostic value of *TPO* and *AOX1* in PTC, the correlation between gene expression and patient survival was analyzed using Kaplan–Meier plotter. We found that low expression of *TPO* and *AOX1* may predict poorer survival in PTC. The Kaplan–Meier plotter is a widely used database for assessing the effects of 54,000 genes on survival in 21 cancer types (19). The primary purposes of the tool are the meta-analysis–based discovery and validation of survival biomarkers (46). Many genes have been explored and/or recognized by Kaplan-Meier plotter in several tumor types (47-49). The database contains gene expression data, clinicopathological information, and RFS information for 502 patients with PTC. In a long-term follow-up study, *TPO* was reported as a prognostic factor for differentiated thyroid cancer (50). Consistent with our results, the expression of *AOX1* was downregulated in prostate cancer, and this lower expression portended a poor prognosis (51). The excellent predictive ability in our study suggested that TPO and AOX1 might be potential prognostic markers for patients with PTC.

In conclusion, independent datasets and experimental validation demonstrated that the expression of *TPO* and *AOX1* in tissue and their protein concentrations in blood plasma were significantly reduced in pediatric PTC. Diagnostic analysis revealed that TPO and AOX1 had excellent predictive performance, indicating their potential usefulness for clinical diagnosis. Survival analysis confirmed that *TPO* and *AOX1* can be used as prognostic biomarkers for pediatric PTC, with low expression predicting worse outcomes in patients. This study revealed that *TPO* and *AOX1* might be promising non-invasive biomarkers for PTC diagnosis and prognosis. Additional studies are needed to explore the mechanisms of *TPO* and *AOX1* in pediatric PTC.

Footnote

Reporting Checklist: The authors have completed the REMARK reporting checklist. Available at http://dx.doi.org/10.21037/tp-20-301

Data Sharing Statement: Available at http://dx.doi.org/10.21037/tp-20-301

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tp-20-301). The authors have no conflicts of interest to declare.

Ethical Statement: The author is accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study follows the Helsinki Declaration (as revised in 2013) and was approved by the Ethics Committee in Beijing Children's Hospital, Capital

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Medical University (2020-k-168). Informed consent was provided by each patient and/or his/her legal guardians before samples were collected and stored in the biobank.

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