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PRDX4 Potentially Predicts the Postoperative Outcome in Advanced Papillary Thyroid Carcinoma

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Abstract: Background: Peroxiredoxin 4 (PRDX4), a secreted antioxidant enzyme, can protect against hepatocellular carcinoma and lung adenocarcinoma, but its role in papillary thyroid carcinoma (PTC) is still unclear. In this study, we investigated the association of the PRDX4 expression with the prognosis of patients with advanced PTC. Methods: We conducted a retrospective case-control study at Kanazawa Medical University Hospital. We selected PTC patients over 55 years of age who received surgery from 2006 to 2014. The PRDX4 expression was immunohistochemically analyzed in paraffin-embedded tumor specimens of 70 patients with stages II–IV advanced PTC. We also investigated the key roles of PRDX4 in a human PTC cell line (K-1) in vitro. Result: The weak expression of PRDX4 was found to be significantly associated with recurrence. In a multivariate analysis, the weak expression of PRDX4—rather than other pathological features of high invasiveness—predicted a poor prognosis. In vitro, the viability of human PTC cells was significantly suppressed after PRXD4 plasmid transfection. Conclusion: The weak expression of PRDX4 can predict recurrence with a potential poor prognosis in advanced PTC.

Keywords: advanced PTC; PRDX4; recurrence; prognosis; cell proliferation

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1. Introduction

Papillary thyroid carcinoma (PTC) is a common malignant tumor. Worldwide, its incidence is increasing each year, especially in young people. In general, early-stage differentiated thyroid cancer has a very good prognosis with a five-year survival rate of >90%. However, when the disease progresses to tumor-node-metastasis (TNM) stage ≥ II, recurrence and distant metastasis become more frequent, and the survival rate drops dramatically [1–4]. Furthermore, the prognosis of patients with advanced PTC varies greatly, with some patients experiencing repeated metastatic recurrence after surgery. Thus far, no significant molecular/genetic factors that predict the postoperative prognosis have been identified. It is therefore very important to find a biomarker that predicts the prognosis of patients with advanced PTC [5–7].

Reactive oxygen species (ROS) play important roles in host defense, cell proliferation, gene expression, and other processes; however, excessive amounts induce oxidative stress, which damages DNA [8,9]. Follicular thyroid cells require H_2O_2 for thyroid hormone synthesis and therefore have oxidative properties, which make them vulnerable to the harmful effects of oxidative stress. It has already been demonstrated that enhanced oxidative stress can lead to the development of thyroid cancer [10–14]. However, there is

a protective system—mainly involving antioxidant enzymes—against oxidative stress in the living body. It has been newly discovered that Peroxiredoxins (PRDXs) play a role in the elimination of toxicity by reducing H_2O_2 generated to H_2O in the body and suppress oxidative stress [15].

Many studies have demonstrated the pivotal roles of PRDXs in various kinds of human cancers, including esophageal, lung, breast, and skin cancer [16–19]. The PRDX family is also expressed in the normal thyroid gland and has been reported to be associated with the development of malignant tumors [20–23]. Among the six types of PRDX, PRDX4 is expressed as the only exocrine form of all antioxidants, eliminating oxidative stress in the extracellular space, and protecting against tissue damage [24]. Like other antioxidant stress factors, PRDX4 has been shown to influence processes of tumor progression, such as infiltration and metastasis in many malignant tumors [25–31].

In this study, using immunohistochemistry, we observed the protein expression of PRDX4 in advanced PTC tissues and evaluated the differences in clinicopathological indexes and the prognosis of advanced PTC between strong and weak PRDX4 expression groups. Further, in vitro, we also investigated the effect of PRDX4 in cell behavior by transfecting plasmid DNA into a PTC cell line (K-1). This study is of great significance to the search for new biomarkers to adjust the therapeutic strategy and improve the prognosis of advanced PTC.

2. Methods

2.1. Patients and Pathological Specimens

One hundred fifty-two patients with PTC who underwent head and neck surgery from 1 April 2006, to April 2014, among whom 70 patients were diagnosed with stages II–IV PTC according to AJCC/UICC 8 [32,33] (the median postoperative observation period was 96.8 Month), were included in the present study. Patients were enrolled after receiving approval from the Ethics Committee of Kanazawa Medical University. Two pathologists evaluated all specimens according to the WHO Classification of Tumors of Endocrine Organs (4th edition) IARC [34].

The following items were evaluated: (1) tumor diameter, (2) presence or absence of vascular infiltration (vein/lymphatic vessel), (3) degree of peripheral infiltration, and (4) presence or absence of intraglandular metastasis. Specimens were subjected to hematoxylin and eosin staining, and Elastica Van Gieson (EVG) staining was also performed if needed. Clinical information was retrospectively analyzed based on the data collected from medical records. The recurrence-free period was defined as the period from the date of surgery to the development of local recurrence, cervical lymph node recurrence, or distant recurrence. Recurrence was assessed by cervical ultrasound echo, computed tomography (CT), positron emission tomography (PET-CT), and magnetic resonance imaging (MRI). Follow-up was performed every 2–4 months for 1 year after the operation, and imaging tests were performed every 6 months after the operation. Additional tests were performed if there were signs of recurrence. During the observation period, only one person died of the primary disease. The survival rate was not evaluated.

2.2. Dispensing/Immunostaining for PRDX4 and Secondary Antibody

Immunostaining was performed using a PRDX4 IgG rabbit polyclonal antibody. A previously described preparation method was used as a reference [35,36]. This PRDX4 antibody was a generous gift from Prof. Junichi Fujii (Department of Biochemistry and Molecular Biology, Graduate School of Medical Science, Yamagata University). Immunostaining was performed by the antibody-bound dextran polymer method using hematoxylin counterstaining. Deparaffinized and rehydrated 4 μm sections were incubated with 10% hydrogen peroxide for 5 min to block endogenous peroxidase activity. The sections were then washed and incubated with PRDX4 IgG rabbit polyclonal antibody (diluted 1:1000) for 2 h.

The secondary antibody peroxidase-binding polymer was then used, and sections were counterstained. Then, the preparations were observed under a light microscope.

2.3. Intensity of Immunostaining

The intensity of the PRDX4 expression was evaluated semi-quantitatively by evaluating the proportion of staining-positive cells. All histological and immunohistochemical slides were evaluated by two independent observers (certified surgical pathologists in our department; A.S. and X.G.) using a blind protocol design (observers blinded to the clinicopathological data). The agreement between the observers, as measured by the interclass correlation coefficient, was excellent (>90%) for the investigated PRDX4 antibody. In the few (<1%) instances of disagreement, a consensus score was determined by a third board-certified pathologist (S.Y.) in our department. A receiver operating characteristic (ROC) curve analysis was performed and the cut-off value was set according to the area under the curve (AUC).

2.4. Cell Culture

The PTC cell line (K-1 cells) was obtained from the JCRB Cell Bank. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum in a humidified environment of 95% air/5% CO_2 at 37 °C.

2.5. Cell Transfection

The PRDX4 plasmid DNA was obtained from the Faculty of Pathology, Kagoshima University. Cells were seeded in 6-well plates, cultured in growth medium to approximately 60% confluence, incubated for 24 h, and transfected with 5 μg of PRDX4 plasmid DNA for 72 h in Opti-MEM medium with Lipofectamine® 2000. (Life Technologies, Carlsbad, CA, USA). PMCV-Tag-2b (kindly gifted by Kagoshima University Faculty of Pathology) vectors were used as negative controls.

2.6. Western Blotting

Proteins were extracted from K-1 cells and separated using SDS polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were equilibrated in transfer buffer and transferred onto a PVDF Western blotting membrane (Roche Diagnostics GmbH, Mannheim, Germany) using a semi-dry rotor. The transferred membrane was blocked with 5% skim milk for 1 h. It was then incubated with the primary antibody for 1 h at room temperature.

The following primary antibody and diluent were used: anti-PRDX4 antibody (1:1000; Invitrogen, Thermofisher, Waltham, MA, USA) (rabbit polyclonal antibody), anti-β-actin, and peroxidase conjugated (1:1000; Wako, Osaka, Japan) (rabbit monoclonal antibody). After washing with the primary antibody, the membrane was exposed to the secondary antibody and incubated. The protein expression was detected with Clarity Western ECL Substrate (Bio-Rad, Berkeley, CA, USA).

2.7. Cell Proliferation Assay

Viable cell counts were measured using the CCK-8 method according to the manufacturer's instructions. A K-1 cell suspension at 10^4 cells/mL was seeded into a 96-well plate, 100 μL per well. Absorbance at 450 nm of 0, 24, 36, and 72 h was measured using a microplate reader.

2.8. Statistical Analysis

First, the significance of the PRDX4 expression intensity relationship was determined using Fisher's exact test. In addition, we evaluated whether independent factors significantly influenced 5-year recurrence-free survival (RFS) by a log-rank test using the Kaplan–Meier method. Second, the hazard ratios of the PRDX4 weak expression group and other factors that are thought to affect recurrence were calculated by a Cox hazards regression analysis. Third, a multivariate analysis was performed using all the items that were included in the above-mentioned univariate analysis as explanatory variables, to examine whether the expression of PRDX4 was useful for predicting recurrence. All statis-

tical analyses were performed using the R software program (version 3.2.3). All statistical analyses were two sided, and statistical significance was defined as $p < 0.05$.

3. Results

3.1. Patient Characteristics

In AJCC/UICC version 8, patients under 55 years of age without distant metastasis are classified as Stage I, and the younger patients did not have distant metastasis, so all the subjects in this study were older than 55 years of age (Table 1). The median age of the patients was 66 years; the oldest was 80 years of age. There were 57 women, accounting for 81% of the total population, and 69% of the cases were classified as Stage II. Eight (11%) patients had distant metastasis at the initial treatment stage, while 40 (57%) had lymph node metastasis. All treatments were curative. Paratracheal dissection was performed in all cases, and cervical dissection at the site of metastasis was performed in patients with lymph node metastasis. For patients with preoperative distant metastasis or postoperative recurrence of distant metastasis were treated with radioactive iodine (RAI, also called I-131) as postoperative treatment. As a result, of the 10 patients who underwent RAI, 6 were considered to be RAI refractory. Pathological findings showed a median tumor size of 19.5 mm (19.5 ± 15.2 mm), 90% showed extracapsular extension, and nearly half of those showed vascular invasion (n = 32 (46%)) or lymphatic permeation (n = 35 (50%)). All histopathological images were highly differentiated, with no evidence of specialization or hypo differentiation. The median recurrence-free period was 63.3 months. Postoperative recurrence was observed in 11 patients; all had lymph node metastasis, while 2 had distant metastasis to the lung.

Table 1. The clinicopathological characteristics of the patients.

Characteristic	Patients (n = 70)
Age	
Median	66
Range	55–80
Sex	
Male	13
Female	57
Clinical Stage	
II	48
III	17
IV	5
Distant metastasis	8
Regional metastasis	40
Operative Method	
Lobectomy	24
Subtotal	3
Thyroidectomy	43
Recurrence	11
Local	0
Regional	11
Distant	2
Pathological	
Tumor size, mm	
Median	19.5
Range	1.2–100
Vascular invasion	32
Lymphatic invasion	35
Extension	
0	7
1	45
2	18
Multifocality	27

3.2. Relationship between the PRDX4 Expression Rate and Clinicopathological Variables and Disease-Free Recurrence Period

The cut-off value for the PRDX4 expression rate was set based on an ROC curve analysis (Figure 1). The association between the PRDX4 expression rate (Table 2 and Figure 2) and clinical pathological features was analyzed using Fisher's exact test (Figure 2). The cut-off value for the PRDX4 expression rate was set to 30%, and the groups were separated into a strong expression group and a weak expression group. There were no differences between strong and weak groups (or two groups or these groups) in age, sex, presence or absence of cervical lymph node metastasis and distant metastasis at the initial treatment, or pathological factors (tumor diameter, venous infiltration, lymphatic vessel infiltration, extracapsular extension, intraglandular metastasis). Postoperative recurrence was the only factor that was significantly associated with the expression of PRDX4 ($p < 0.05$). Figure 3 shows the transition of 5-year RFS in two groups with a cut-off value of 30% using the Kaplan–Meier curve. There was a significant (≥5%) difference in 5-year RFS between the two groups ($p < 0.05$).

Table 2. Detailed correlations between the PRDX4 expression and clinicopathological variables.

Variable, *n* (%)	Weak Expression (*n* = 32)	Strong Expression (*n* = 38)	*p*
Gender			
Male	7 (22)	6 (16)	0.55
Female	25 (78)	32 (84)	
Tumor size			
>20 mm	22 (69)	28 (74)	0.79
≤2 mm	10 (31)	10 (26)	
Vascular invasion			
Positive	11 (34)	21 (55)	0.1
Negative	21 (66)	17 (45)	
Lymphatic invasion			
Positive	13 (41)	22 (58)	0.23
Negative	19 (59)	16 (42)	
Extension			
Positive	3 (9)	4 (11)	1
Negative	29 (91)	34 (89)	
Multifocality			
Positive	13 (41)	10 (26)	0.31
Negative	19 (59)	28 (74)	
Regional metastasis			
Positive	13 (41)	18 (47)	0.63
Negative	19 (59)	20 (53)	
Distant metastasis			
Positive	4 (14)	4 (8)	1
Negative	28 (86)	34 (92)	
Recurrence			
Positive	9 (18)	2 (5)	0.02
Negative	23 (72)	36 (95)	
Stage			
II	29 (76)	19 (59)	0.20
III, IV	9 (24)	13 (41)	

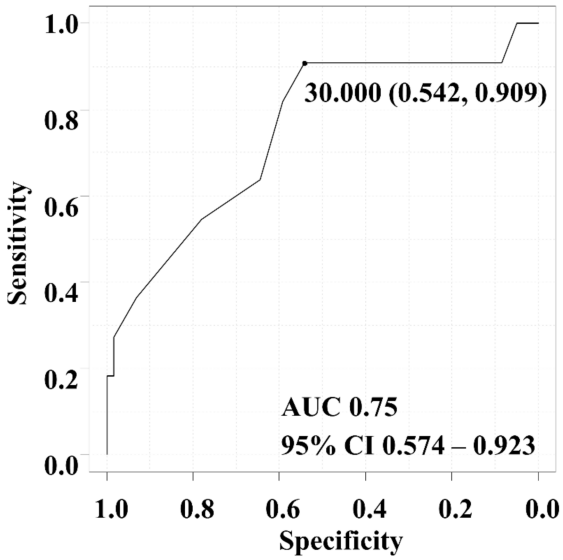

Figure 1. Receiver operating characteristic (ROC) curve analysis was used to select and validate the cut-off value for the immunohistochemical expression of PRDX4. The cut-off value was selected using a ROC and the area under the curve (AUC). The cut-off value was 30%.

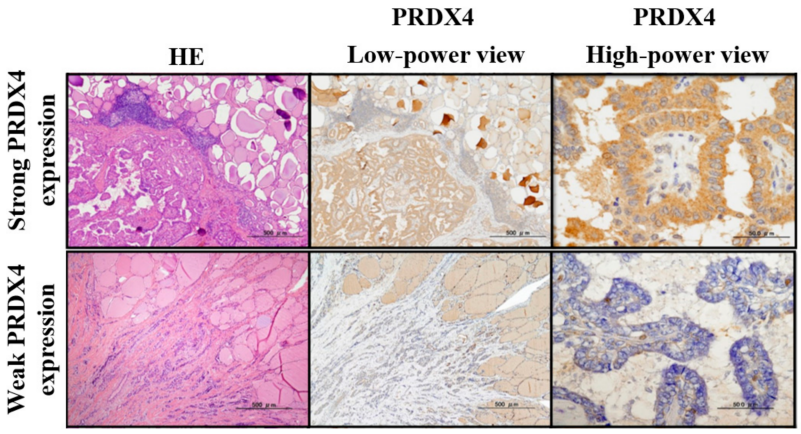

Figure 2. The immunohistochemical analysis of PRDX4 in PTC. Representative images of hematoxylin eosin (HE) staining (left) and the immunohistochemical analysis of PRDX4 (middle and right) in stage II-IV thyroid papillary carcinoma (strong PRDX4; weak PRDX4). Intracytoplasmic staining pattern of PRDX4 was confirmed in papillary thyroid carcinoma cells (left and middle: Bar = 500 μm; right: Bar = 50 μm).

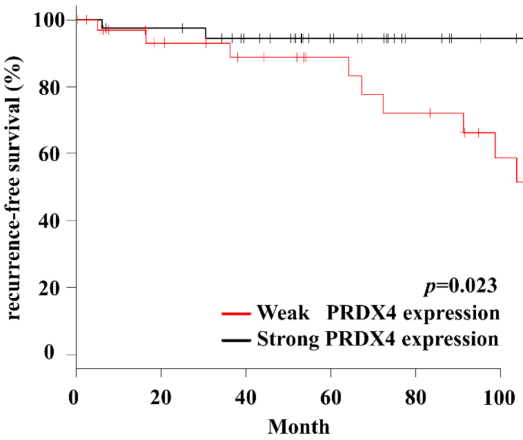

Figure 3. Kaplan–Meier curve for recurrence-free survival (RFS) in patients after PTC surgery according to the expression of PRDX4. Table 3 was associated with significantly shorter postoperative RFS in PTC patients.

3.3. Cox Proportional Hazards Regression Analysis

A Cox proportional hazard regression analysis was performed to assess whether the expression of PRDX4 was an independent predictor of postoperative RFS. The analysis included pathological factors associated with a risk of recurrence of PTC. Table 3 shows the results of the univariate and multivariate analyses. In the univariate analysis, the presence or absence of distant metastases at the time of initial treatment and the weak expression of PRDX4 were identified as significant factors, with hazard ratios of 5.85 and 4.88, respectively. When all the variables in the univariate analysis were used as explanatory variables in a multivariate analysis, the weak expression of PRDX4 was the only significant factor, suggesting that it is an effective recurrence predictor (HR7.52, 95%CI 1.23–42.6, $p = 0.02$).

Table 3. Univariate and multivariate analyses of survival according to the PRDX4 expression labelling index.

	Univariate			Multivariate		
	HR	**95% CI**	***p*-Value**	**HR**	**95% CI**	***p*-Value**
PRDX4 < 30%	4.88	1.04–22.8	0.04	7.52	1.24–42.6	0.02
Vascular invasion	1.72	0.52–5.67	0.36	9.00	0.23–358.8	0.24
Lymphatic invasion	1.26	0.38–4.12	0.71	0.14	0.003–5.94	0.31
Tumor diameter > 2 cm	2.95	0.90–9.68	0.07	6.96	0.90–53.7	0.06
Extrathyroid > 1	1.21	0.32–4.56	0.78	0.10	0.009–1.17	0.07
Regional metastasis	1.48	0.39–5.66	0.57	2.18	0.39–12.2	0.38
Distant metastasis	5.85	1.39–24.7	0.02	4.04	0.73–22.3	0.11
Multifocality	1.29	0.38–4.30	0.69	0.57	0.12–2.66	0.48

3.4. The Expression of PRDX4 and Cell Proliferation Ability

After transfection of PRDX4 plasmid DNA and negative vector into K-1 cells, the cell proliferation ability was analyzed for 3 days by the CCK-8 method. The cell proliferation ability of the PRDX4 plasmid DNA transfection group was found to be significantly suppressed (Figure 4).

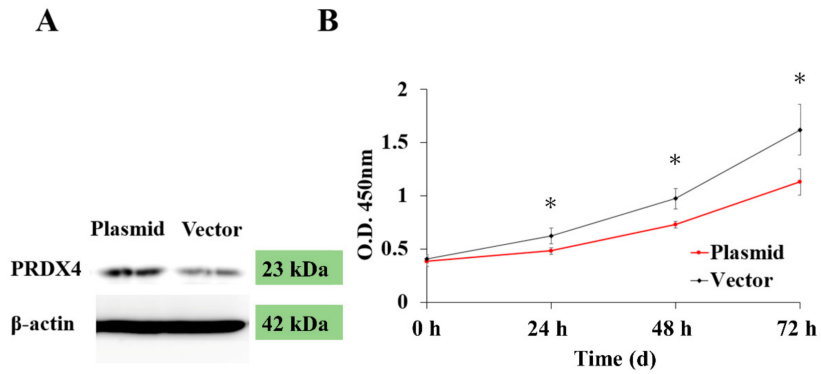

Figure 4. Western blotting and cell proliferation A: The protein expression of PRDX4 was remarkably increased after transfection of PRDX4 plasmid in K-1. B: The proliferation of K-1 cells was analyzed using a CCK-8 kit, 3 days after the transfection of PRDX4 plasmid DNAs or negative vectors. Plasmid, PRDX4 plasmid DNA; Vector, negative control vector; * *t*-test, $p < 0.05$.

4. Discussion

Oxidative stressors play a major role in the process of thyroid hormone formation [10]. When thyroid follicular cells make thyroid hormone, they make large amounts of H_2O_2 and accumulate in the follicular lumen or colloid. Therefore, the thyroid gland is always exposed to oxidative stress factors, but some Selenium (Se)-containing antioxidant factors in the thyroid cells reduce intracellular oxidative stress to balance ROS production and excretion [35]. Other than typical antioxidant factors (e.g., glutathione peroxidase, thiore-

doxin, and reductase families) PRDXs are important factors in maintaining homeostasis within the thyroid gland. The levels of PRDX1 and PRDX6 in PTC tissue are significantly reduced in comparison to normal tissue, and an increase in the expression of PRDX5 was observed in thyroid tumor cells, thus indicating that—in the thyroid cells—PRDXs play important protective roles against the negative effects of oxidative stress [20–23]. The effect of the PRDXs expression on the growth and prognosis of PTC remains unclear.

In this study, our results showed that the weak expression of PRDX4 in PTC cells may be associated with postoperative recurrence and the 5-year RFS in PTC with the weak expression of PRDX4 decreased by $\geq$5%, suggesting that PRDX4 could be an independent predictor of postoperative recurrence. Predicting recurrence in advance is very important because recurrence has a significant impact on survival in PTC [1–4]. These results further confirm that PRDX4 plays a protective role in PTC tissues. Therefore, for those patients with advanced thyroid tumor showing low PRDX4 ex-pression, postoperative follow-up should be strengthened, so that the disease recurrence can be found as soon as possible, so as to avoid the serious consequences caused by distant metastasis or other complications. In PTC cells, intracellular oxidative stress factors are significantly increased, but antioxidant factors will increase or decrease depending on their characteristics [10–14]. The antioxidation mechanism of PRDX4 in PTC is not clear, and it therefore needs to be further clarified in a future study. Moreover, our previous study also confirmed the association between the PRDX4 expression and EGFR mutation status, suggesting that EGFR mutations affected the role of PRDX4 in the proliferation of LUAD cells. Thus, the present results compared with the TCGA cohort may more comprehensively understand its role in PTC in future research.

It should be noted that the effects of PRDX4 are not always consistent in malignancies. In advanced prostate cancer, the weak intracellular expression of PRDX4 has been shown to be associated with a significantly worse prognosis [27]. Our previous study also demonstrated that in hepatocellular carcinoma, hepatoblastoma, and lung adenocarcinoma cells, PRDX4 weak expression groups showed faster cell proliferation and a poor prognosis [36–39]. These observations are consistent with our results in the present study. On the other hand, in oral squamous cell carcinoma and urethane-induced lung adenocarcinoma, the overexpression of PRDX4 promotes the proliferation of malignant tumor cells and has the opposite effect to worsening the prognosis [28,29]. These discrepancies may be due not only to the histological types of the malignancies but also to the methods of evaluation in each study, for example the size of the cohort, the use of different antibodies, or the selection and validation of cut-off scores for PRDX4.

Moreover, during the inhibition of cancer progression, PRDX4 also plays different functions in different types of malignancies. PRDX4 suppresses cell proliferation and migration in hepatocellular carcinoma, while it induces cell differentiation in hepatoblastoma [38,39]. In this study, when the PRDX4 plasmid DNAs were transfected into K-1 cells in vitro, the proliferative capacity of these cells was significantly suppressed by the strong expression of PRDX4, confirming that PRDX4 can affect PTC cell proliferation by regulating the tumor microenvironment. Therefore, we speculate that it improved the microenvironment of tumor growth by regulating intracellular and extracellular redox balance, so as to inhibit cell proliferation and further reduce the recurrence of the disease. Further experiments, including cell migration and invasion, are also needed to confirm the specific mechanism in future studies.

The present study was associated with some limitations. Firstly, a bias may have been present due to the single-center, retrospective cohort design. It is necessary to conduct prospective studies at other facilities in the future. Secondly, regarding the consistency of treatment, in Japan, additional treatment is given if distant metastasis or high-risk factors are identified before surgery [6]. Patients will receive postoperative irradiation therapy as additional treatment according to guidelines [40,41]. As a result, the postoperative course may be affected. Finally, although the median postoperative follow-up period in this study exceeded 5 years, it would still be desirable to examine the 10-year survival rate because the 5-year survival rate for papillary thyroid cancer is >90%, even for advanced cancer.

Moreover, more scientific measures of follow-up are needed [42]. Since no patients died of the primary disease in this study, we could not examine the survival rate [1–4]. It is expected that clearer results will be detected by observing the patients for >10 years in the future.

5. Conclusions

This study suggests that the weak expression of PRDX4 in PTC tissue is closely associated with postoperative recurrence, suggesting that this antioxidant enzyme may be an independent prognostic factor for postoperative recurrence of advanced PTC.

Author Contributions: Conceptualization: Y.T., X.G., J.H. and S.Y.; Data Curation: Y.T., X.G., J.H. and S.Y.; Formal Analysis: Y.T., X.G., J.H. and S.Y.; Funding Acquisition: Y.T., X.G. and S.Y.; Investigation: Y.T., X.G., J.H., A.S., Y.S., Y.K., M.K., H.T. and S.Y.; Methodology: Y.T., X.G., J.H., A.S., Y.S., Y.K., M.K., H.T. and S.Y.; Project Ad-ministration: Y.T., X.G., J.H., A.S., Y.S., Y.K., M.K., H.T. and S.Y.; Resources: X.G. and S.Y.; Software: Y.T., X.G., J.H., A.S., Y.S., Y.K., M.K., H.T. and S.Y.; Supervision: X.G., J.H. and S.Y.; Validation: Y.T., X.G. and S.Y.; Visualization: Y.T., X.G., J.H. and S.Y.; Writing Original Draft Preparation: Y.T., X.G. and J.H.; Writing Review and Editing: Y.T., X.G., J.H. and S.Y. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of Kanazawa Medical University (protocol code I695 and date of approval) for studies involving humans.

Informed Consent Statement: Written informed consent was obtained from the patient the patient and his family on admission for the publication of this case report and any accompanying images.

Data Availability Statement: The dataset supporting the findings and conclusions of this research is included within the article.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

PRDX4: peroxiredoxin4; TNM, tumor-node-metastasis; ROS, reactive oxygen species; HCC, hepatocellular carcinoma; HB, hepatoblastoma; LUAD, lung adenocarcinoma; EGFR, epidermal growth factor receptor; EVG, Elastica Van Gieson; CT, computed tomography; PET-CT, positron emission tomography; MRI, magnetic resonance imaging; ROC, receiver operating characteristic; AUC, area under the curve; K-1 cell, PTC cell line; DMEM, Dulbecco's Modified Eagle Medium; SDS-PAGE, SDS polyacrylamide gel electrophoresis; OS, overall survival; DFS, Disease-free survival; RFS, recurrence-free survival.

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