Correlation between low expression of protein disulfide isomerase A3 and lymph node metastasis in papillary thyroid carcinoma and poor prognosis: a clinicopathological study of 1,139 cases with long-term follow-up

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Abstract. The incidence of papillary thyroid carcinoma (PTC) is increasing worldwide. The biomarkers to identify aggressive types of PTC are limited, illustrating the need to establish reliable novel biomarkers. Protein disulfide isomerase A3 (PDIA3) is a chaperone protein that modulates the folding of newly synthesized glycoproteins and stress-responsive proteins in the endoplasmic reticulum. Although the role of PDIA3 in various cancers such as breast, uterine cervix, head and neck, and gastrointestinal tract has been examined, its expression in thyroid cancer has not been reported. We retrospectively reviewed accumulated data with long-term follow-up of 1,139 PTC patients, and investigated the correlation between immunohistochemical expression of PDIA3 in PTC patients and clinicopathological features and prognosis. PDIA3 expression was significantly lower in PTCs compared to normal thyroid tissues (NTT; \(n = 80, p = 0.002\)). In PTCs, correlation between low PDIA3 expression and lymph node metastasis \((p = 0.018)\) and the number of positive nodes \((p = 0.004)\) was observed. Patients with low PDIA3 expression exhibited worse cause-specific survival compared to those with high PDIA3 expression \((p = 0.013)\). Our findings indicate that low PDIA3 expression is related to poor clinical outcome in PTC patients, and that PDIA3 may potentially be a novel ancillary biomarker. Further clarification of the biological role of PDIA3 in PTC is warranted for the future clinical application.

Key words: Papillary thyroid carcinoma (PTC), Protein disulfide isomerase A3 (PDIA3), Immunohistochemistry, Lymph node metastasis (LNM), Prognosis

THYROID CANCER is one of the most common malignancies of the endocrine system and its incidence is rapidly increasing worldwide [1]. Among thyroid cancers, papillary thyroid carcinoma (PTC) is the most common histological type. Although PTC generally has an indolent and slow, progressive behavior, 5–10% of PTC patients experience a more aggressive clinical course, characterized by recurrent disease and early metastases, and resistance to radioactive iodine, resulting in increased mortality [2]. Aggressive clinicopathologic features of PTC such as large tumor size, multifocality, massive extrathyroidal extension, lymph vascular invasion, large or extranodal invasive lymph node metastasis (LNM), distant metastasis, histological subtypes, and TERT promoter mutation have been reported [3-6]. Despite these facts, biomarkers to identify aggressive types of PTC are limited, illustrating the need to establish reliable novel biomarkers.
Protein disulfide isomerase A3 (PDIA3), also known as ERp57/GRP58, is of the protein disulfide isomerase family. The functions of PDIA3 in the endoplasmic reticulum (ER), including the proper folding and quality control of glycoproteins as well as participation in the assembly of major histocompatibility complexes (MHC) class I are well known [7]. The roles of PDIA3 in tumors vary depending on the tumor type. To date, PDIA3 has been reported to be involved in the following tumors: breast [8], uterine cervical cancer [9], gastric [10], hepatocellular [11, 12], colorectal [13], lung [14], laryngeal carcinoma [15], and glioma [16]. In some of these tumors, high expression is correlated with poor prognosis [11, 12], while in others it is correlated with good prognosis [9, 10]. The involvement of PDIA3 in thyroid cancer is still unknown.

Here, we investigated the immunohistochemical expression of PDIA3 in 1,139 PTC patients with long-term follow-up (mean, 12 years) at a single institution in order to identify its correlation with other clinicopathological features and prognosis.

Materials and Methods

Case selection

For this retrospective study, patients with PTC who underwent primary thyroid surgery between January 1993 and December 2012 at Cancer Institute Hospital, Japanese Foundation for Cancer Research (JFCR), Tokyo, Japan were targeted. Patients with papillary microcarcinoma (maximal diameter, ≤1.0 cm) were excluded. We designed our own risk group classification for predicting cause-specific death from PTC [5]. That is, patients with distant metastasis and older patients (≥50 years) with massive extrathyroidal invasion or large lymph node metastasis (LNM) (maximal diameter, ≥3 cm) were defined as high risk and all other patients were classified as low risk. According to the risk adapted management treatment approach, we provided treatment options as described in previous reports [4]. Briefly, we basically conducted lobectomy for patients with unilateral low-risk PTC. For patients with high-risk PTC, total thyroidectomy followed by radioactive iodine treatment was recommended. Extent of lymph node dissection was determined as follows [17]: a) Dissection of the central compartment (level VI) alone for patients with LNM only in the central zone or with no LNM; and b) lateral neck dissection (basically, levels II, III, IV, and VI) when the patient was diagnosed with lateral neck LNM. Bilateral neck dissection was performed only when preoperative imaging showed bilateral neck LNM. Patients were observed until December 26, 2018, or death, whichever came first. Cases with small tumor components, and/or severe calcification, were excluded from the study. In addition, 80 cases of normal thyroid tissue (NTT) array samples were purchased from Funakoshi Co., Ltd. (Tokyo, Japan). Patient demographics and clinicopathological data were obtained from medical records. This study was conducted under the principles embodied in the Declaration of Helsinki (revised in Brazil 2013), and was approved by the institutional review boards of Nippon Medical School Hospital (#30-03-1111, June 6, 2020) and of the JFCR (IRB number 2013–1128, on January 24, 2014, last updated on March 23, 2021). Written informed consent for the use of clinical samples for research purposes was obtained from the patients.

Immunohistochemistry

Expression of PDIA3 was evaluated by immunohistochemistry of tissue microarrays (TMAs). We constructed TMA as previously described [4]. In brief, for each tumor, the experienced, boarded pathologists selected one-to several representative histological areas for tissue microarray. We punched the selected these points of the donor paraffin blocks with a 2 mm diameter coring needle, and transferred the tissue to the array in the recipient block using a manual tissue arrayer (KIN-1; Azumaya, Tokyo, Japan). Tissue sections with a 4 μm thickness were used for immunostaining. Following deparaffinization, sections were pretreated at 95℃ for 40 min in 10 mM citrate buffer solution (pH 6.0). Endogenous peroxidase was blocked in 100% methanol (Wako Pure Chemical Industries, Ltd.) containing 0.3% hydrogen peroxide (Wako Pure Chemical Industries, Ltd.) for 30 min at room temperature. The sections were then incubated with an anti-PDIA3 mouse monoclonal antibody (CL2444, Invitrogen/Thermo Fisher Scientific K.K., Tokyo, 1:10,000 dilution), using the Nichirei Histostainer (Nichirei Biosciences INC., Tokyo, Japan). The sections were further incubated with Histofine Simple Stain MAX PO (mouse; Nichirei Biosciences, Inc., Tokyo, Japan) for 30 min at room temperature, and peroxidase activity was visualized by 3,3’ dianimobenzidine. The sections were then counterstained with hematoxylin at room temperature for 1 min.

Immunohistochemical analysis

Intensity and ratio of stained carcinoma cells were scored using a semi-quantitative method. The slides were scanned using Nano Zoomer 2.0-HT (Hamamatsu Photonics K.K., Tokyo, Japan) and analyzed using QuPath software v0.2.3 [18]. Cytoplasmic staining was considered to indicate a positive reaction. The expression of PDIA3 was determined using the H-score. H-score was determined by the sum of (each category of cytoplasmic intensity from 0 to 3) × (percentage of tumor
The results ranged from 0 to 300, where 0 indicates all cells are negative, and 300 indicates all cells are strongly positive. The QuPath software analysis was performed twice, and the average score was calculated. Then, the average score for several cores from each case was determined as H-score of the case. The cases were then divided into high and low groups depending on whether they were above and below the median score, respectively.

**Statistical analyses**

All categorical variables are presented as numbers and percentages, while continuous variables are presented as mean ± standard deviation. H-scores in the immunohistochemical analysis were compared using Mann Whitney’s U test. Clinicopathological parameters were analyzed using the Mann-Whitney’s U test, and Chi-square test. Cause-specific survival (CSS) time was measured from the date of diagnosis to the date of cause-specific death or the date of the last follow-up. Disease-free survival (DFS) time was measured from the date of diagnosis to the date of the first relapse or the date of the last follow-up. Cox proportional hazards model was used to identify independent factors which significantly influenced survival. The probability of CSS and DFS were calculated using the Kaplan-Meier method and compared using the log-rank test. Statistically significant difference was set at $p < 0.05$. All statistical analyses were performed using SPSS (version 26; IBM Corp., Armonk, NY, USA).

**Results**

**Clinicopathological characteristics of PTCs**

Of the 1,435 patients in our archives, clinical data and tissue of 1,139 PTC patients were available for analysis. Baseline clinicopathological characteristics of PTC patients are summarized in Table 1. Targeted patients were comprised of 873 women and 266 men with a mean age of 52.3 ± 13.9 years. Mean tumor size was 24.8 ± 14.9 mm. According to the AMES (age, metastasis, extrathyroidal extension) [19] risk classification systems, 947 patients (83%) were classified as low-risk, and 192 patients (17%) as high-risk. When applying the Cancer Institute Hospital risk classification group [5], 944 patients (83%) were classified as high-risk, and 195 patients (17%) were classified at low risk. The mean follow-up period was 12 years (range 154 days–25 years). During the follow-up period, 957 patients (84%) were alive without recurrent cancer, 98 patients (9%) were alive with residual or recurrent cancer, and 84 patients (8%) died of recurrent cancer. Mean CSS time was 23.8 years (95% CI 23.5–24.3 years). DFS time was 21.8 years (95% CI 21.3–22.3).

**Table 1 Baseline characteristics of the patients**

| Factors                                    | Cases (n = 1,139) |
|--------------------------------------------|------------------|
| Age                                        | 52.3 ± 13.9      |
| Sex                                        |                  |
| Female                                     | 873 (77%)        |
| Male                                       | 266 (23%)        |
| Mean tumor size ± S.D. (mm) (range)        | 24.8 ± 14.9 (11–130) |
| Histological subtypes                      |                  |
| Conventional type                          | 985 (87%)        |
| Follicular variant                         | 152 (13%)        |
| Diffuse sclerosing variant                 | 2 (0.2%)         |
| Extrathyroidal extension (pathological)    |                  |
| Absent                                     | 530 (47%)        |
| Present                                    | 609 (53%)        |
| LNM (pathological)                         |                  |
| Absent                                     | 643 (56%)        |
| Present                                    | 496 (44%)        |
| Maximum size of LNM                        |                  |
| Mean ± S.D. mm (range)                     | 27.6 ± 14.7 (9–105) |
| LNM size                                   |                  |
| ≤30 mm                                     | 1,000 (88%)      |
| >31 mm                                     | 139 (12%)        |
| Number of LNM                              |                  |
| Mean ± S.D. (range)                        | 2.01 ± 4.2 (1–34) |
| Distant metastasis                         |                  |
| Absent                                     | 1,067 (94%)      |
| Present                                    | 72 (6%)          |
| AMES risk classification                    |                  |
| Low-risk                                   | 947 (83%)        |
| High-risk                                  | 192 (17%)        |
| CIH risk classification group [5]          |                  |
| Low-risk                                   | 944 (83%)        |
| High-risk                                  | 195 (17%)        |

AMES, age, metastasis, extent and size; CHI, Cancer Institute Hospital; LNM, lymph node metastasis; S.D., standard deviation.

**The expression of PDIA3 in NTT and PTC**

Significant expression of PDIA3 was diffusely found in the cytoplasm of follicular epithelial cells in NTT, whereas its expression was relatively weak in the cytoplasm of tumor cells in PTC. The H-score of PDIA3 expression ranged between 0 and 300, and the median H-scores of PDIA3 in NTT and PTC were 148.6 and 131.1, respectively, indicative of a decreased PDIA3 expression in PTC ($p = 0.002$) (Fig. 1A). Representative immunohistochemical stainings of NTT and PTC are shown in Fig. 1B–D.
Correlation between PDIA3 expression in PTC and clinicopathological features

PTC cases were divided into PDIA3-low (570 cases) groups and -high (569 cases) depending on whether they were above and below the median score of 131.1. In order to assess the correlation between PDIA3 expression and clinicopathological features (Table 2). The PDIA3-low expression group showed a higher incidence of LNM ($p = 0.018$), and an increased number of positive lymph nodes ($p = 0.004$), compared to the PDIA3-high expression group. The LNM is categorized as central and lateral LNM. The PDIA3-low group showed a higher incidence of lateral LNM, compared to PDIA3-high group (211/268 cases (82%) vs. 171/228 (75%), $p = 0.038$). Nine patients showed anaplastic transformation during follow-up; but no significant correlation between PDIA3 expression and the frequency of anaplastic transformation was observed ($p = 1.000$).

Correlation between PDIA3 expression and clinical outcome

During the follow-up, 56 patients (10%) in the PDIA3-low group and 28 patients (5%) in the PDIA3-high group died of the disease. In the PDIA3-low group, seven patients died of the local lesions/recurrence, and 49 patients died of metastatic lesions. In the PDIA3-high group, eight patients died of local lesions/recurrence, and 20 patients died of metastatic lesions. Anaplastic transformation was detected in five and four of the dead PDIA3-low and -high groups, respectively.

For patients with no distant metastasis ($n = 1067$), Cox proportional hazard regression analysis of the correlation between clinicopathological features and CSS was performed. Hazard ratios for age, extrathyroidal extension, tumor size >41 mm, LNM >31 mm, and low PDIA3 expression significantly increased according to univariate analysis (Table 3). According to multivariate analysis, age, LNM >31 mm, extrathyroidal extension, and low PDIA3 expression were determined to be independent factors for CSS (Table 3). According to log-rank test, CSS of the PDIA3-low group was significantly worse than that of the PDIA3-high group (23.9 years, 95% CI 23.4-24.4 vs. 24.9 years, 95% CI 24.4-25.3 years, $p = 0.013$) (Fig. 2A).

Mean DFS in the PDIA3-low and -high groups were 23.3 years and 20.1 years, respectively. According to

Fig. 1 Immunohistochemical stainings of PDIA3. H-scores between NTT and PTC were compared, and H-scores of PTC were significantly lower than those of NTT ($p = 0.002$) (A). In NTT, follicular epithelial cells exhibited diffuse positive staining for PDIA3 in the cell cytoplasm (B). In PTC, PDIA3 was variably expressed in the cytoplasm of the tumor cells. Representative findings of PTC with the PDIA3-low (C) and -high (D) expressions.

NTT, normal thyroid tissue; PTC, papillary thyroid carcinoma. Original magnification, ×400. Bars, 50 μm.
univariate and multivariate analyses, age, tumor size >41 mm, LNM >31 mm and extrathyroidal extension were independent factors, but PDIA3 expression was not an independent factor (Table 4, Fig. 2B).

Discussion

The accumulated data of 1,139 patients with PTC were retrospectively reviewed, and expression of PDIA3
assessed. In the current large-scale population with a long-term follow-up study, correlation between low expression of PDIA3 in PTC and incidence of LNM, the number of positive nodes, and poor CSS were observed. The role of PDIA3 is complex and disease dependent, and, to date, no study has investigated PDIA3 expression in thyroid cancer. This is the first report to demonstrate a correlation between a low expression of PDIA3 in PTC and its clinicopathological features.

PDIA3 is a member of the PDI family and was first described as a stress-responsive protein, which is up-regulated under the glucose depletion-induced cellular stress [20]. Unlike other members, PDIA3 is not only located in the ER lumen but also in many subcellular locations, as it does not contain a C-terminal ER retention motif [21]. PDIA3 is expressed ubiquitously at the systemic level, but is present in various types of tissues at varying degrees. In this study, PDIA3 was identified in the cytoplasm of normal thyroid follicular epithelial cells as well as in tumor cells of PTC. These results suggest that possible localizations within thyroid cells should be in the ER, mitochondria, and cytosol. In the ER, biological functions of PDIA3 are reported to be protein folding [22], stabilization of MHC class I [23], and calcium homeostasis [24]. PDIA3 modulates apoptotic signaling [25, 26] and calcium uptake in the mitochondria, actively modulates mTOR complexes [27] and promotes monocyte/macrophage differentiation in the cytosol [28].

PDIA3 has been reported to be related to tumor proliferation and prognosis, but the degree of expression and its functions greatly vary among carcinomas of different sites. Our research group previously reported a higher PDIA3 expression in hepatocellular carcinoma in comparison with normal liver tissue [11]. A higher PDIA3 expression has also been reported for laryngeal cancer [15] and non-small cell lung cancer [14]. On the other hand, expression of PDIA3 has been reported to be inversely correlated with the degree of differentiation in some types of tumors. In uterine cervical cancer, PDIA3 expression was down-regulated compared to cervical intraepithelial neoplasia or normal tissue [9]. In gastric cancer, PDIA3 expression was lower in poorly differentiated components than that in well-differentiated components and normal gastric epithelium [10]. In this study,

Fig. 2 Comparison of CSS (A) and DFS (B) according to PDIA3 expression.
(A) PDIA3-low group exhibited a shorter CSS than that for the PDIA3-high group.
(B) PDIA3 expression was not an independent factor of DFS. DFS did not significantly differ between the PDIA3-low and -high groups.

CSS, cause-specific survival; DFS, disease-free survival.

Table 4 Cox proportional hazard regression analysis of the correlation between clinicopathological features and disease-free survival

| Factors                                | Univariate analysis | Multivariate analysis |
|----------------------------------------|---------------------|----------------------|
|                                        | HR (95%CI)          | p value              | HR (95%CI)          | p value              |
| Age                                    | 1.052 (1.036–1.068) | <0.001               | 1.043 (1.028–1.059) | <0.001               |
| Sex (male vs. female)                  | 0.804 (0.653–0.990) | 0.040                | 0.964 (0.780–1.191) | 0.733                |
| Tumor size (≤40 mm vs. >41 mm)         | 4.953 (3.318–7.395) | <0.001               | 3.440 (2.277–5.198) | <0.001               |
| LNM (≤30 mm vs. >31 mm)                | 1.034 (1.026–1.043) | <0.001               | 3.106 (2.012–4.795) | <0.001               |
| Extrathyroidal extension               | 5.606 (3.383–9.289) | <0.001               | 3.233 (1.907–5.480) | <0.001               |
| PDIA3 (low vs. high)                   | 0.766 (0.525–1.116) | 0.165                | 0.824 (0.563–1.205) | 0.317                |

LNM, lymph node metastasis.
PDIA3 expression was significantly decreased in PTC cells compared to NTT. The roles of PDIA3 in PTC may be due to its multifunction, but the underlying mechanism of a low PDIA3 expression in PTC still remains unsolved.

In this study, a low expression of PDIA3 was significantly correlated with poor CSS in PTC patients. This finding agrees with previous reports on early-stage gastric cancer [10, 29], cervical cancer [9], head and neck cancer [30], and non-small cell lung cancer prognosis [14]. While the underlying mechanism has not been fully elucidated, this may be due to dysfunction of the MHC class I complex, which is stabilized by PDIA3 under the normal conditions [9, 10]. In cancer immunity, PDIA3 forms a complex with MHC class I to facilitate antigen processing, thus activating an immune response against cancer cells [10]. In contrast, when MHC class I expression is insufficient, cancer cells can avoid the cytotoxic effects of immune cells as seen in gastric cancer [31]. Similarly, down-regulation of the MHC class I complex and subsequent acceleration of tumor progression and metastasis has been reported for various tumors, including thyroid cancer [29, 30, 32-34]. Experiments which can clarify the correlation between the molecular mechanism of PDIA3 and MHC class I in thyroid cancer are needed in the future.

The treatment approach including the range of lymph node dissection varies depending on traditional policies and the guidelines. Lobectomy has been widely performed in Japan. On the contrary, treatment teams in Western countries are still apt to choose total thyroidectomy and radioactive iodine therapy. In Japan, According to the Japanese guideline established by Japan Association of Endocrine Surgeons (JAES)/Japanese Society of Thyroid Surgery (JSTS) (the current Japan Association of Endocrine Surgery), lobectomy with prophylactic central lymph node dissection is recommended for low- and intermediate-risk PTC patients [35, 36]. The prophylactic lateral node dissection is only recommended for intermediate- and high-risk patients, with a comprehensive evaluation of risk factors, such as tumor size, distant metastasis and patients’ background and preference. In the guideline of the American Thyroid Association, therapeutic lymph node dissection is well accepted, but prophylactic lymph node dissection is not recommended [2].

In our current study, PDIA3-low was associated with LNM status, especially lateral LNM, and numbers of LNM. Although the p-value is statistically significant, it should be noted that sensitivity and specificity of low PDIA3 in predicting LNM were 54% (95%CI: 49–58%) and 53% (95%CI: 49–56%), respectively, and therefore, low PDIA3 in predicting LNM is associated with 46% false negatives and 47% false positives. Considering the different treatment approaches, the impact of subclinical LNM on prognosis [37], and the potential complications associated with dissection (Chyle leakage, phrenic nerve injury, and accessory nerve paralysis [38]), prophylactic lateral lymph node dissection is not always a required approach. Still, the diagnosis of “cancer recurrence” gives the patients psychological impact [39], and PDIA3 can be one of the ancillary markers for a comprehensive assessment of the lymph node status. Since LNM itself is not considered a strong prognostic factor [37], the question remains that PDIA3 expression in PTC was associated with lymph node status and CSS, but not with DFS. There should be an underlying mechanism to explain this discrepancy, and future experiment is essential.

Regarding limitations to this study, first, all evaluations were carried out using TMA slides. Ideally, evaluation by conventional whole section analysis is desirable. Second, despite that the strength of our study lies in the large cohort treated under a uniform strategy accompanied by long-term, precise outcome information described in the Method section and previous report [5], this study was a retrospective analysis of data prospectively accumulated from a single tertiary oncology referral center in Japan. Both global and prospective verification, in order to confirm these results would be advantageous. Third, we assessed PDIA3 expression only in PTCs of mainly classical type in this study. Further analysis including other histological subtypes—follicular thyroid carcinoma, poorly differentiated thyroid carcinoma, and anaplastic thyroid carcinoma—may help to understand the biological function of PDIA3 in thyroid cancer.

In conclusion, this is the first study to demonstrate that PDIA3 expression is significantly lower in PTC compared to NTT, and that a lower PDIA3 expression is correlated with nodal status, and a poor CSS. These findings suggest that PDIA3 may influence the biological behavior of PTC, although further study to elucidate the underlying mechanism is warranted.

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Disclosure Statement

None of the authors have any potential conflicts of interest associated with this research.

Conflict of Interest

The authors declare no conflict of interest.

Ethics Approval and Consent to Participate

This study was conducted in accordance with the principles embodied in the Declaration of Helsinki (revised in Brazil 2013). Patients’ consents were obtained for the use of clinical samples for research purposes according to the regulations defined by the Ethics Committee of Nippon Medical School Hospital (#30-03-1111, June 3, 2020) and institutional review board of the JFCR (IRB number 2013–1128, 24 January 2014).

Author Contributions

S.K. designed the study. A.E., K. Toda, H.M., and I.S. collected the clinical data. S.K., T.C., A.E., I.S., and K. Toda, reviewed the histopathology of all cases. S.K. and T.C. reviewed the histopathology and immunohistochemistry of all cases and interpreted the results. S.K., T.C., A.E., K. Toda, T.J., N.M., H.M., I.S., K. Takeuchi, and R.O. provided acquisition, analysis and interpretation of data, and statistical analysis. S.K. wrote the initial draft of the manuscript. I.S., K. Takeuchi, and R.O. supervised the study. All authors read and approved the final paper.

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Data Availability Statement

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

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