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ORIGINAL ARTICLE

Increased expression of peroxiredoxin 6 and cyclophilin A in squamous cell carcinoma of the tongue

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OBJECTIVES: The aim of this study was to assess the expression levels of two proteins, such as PRDX6 and cyclophilin A (CypA), and to evaluate their relationship with clinicopathologic features and survival in tongue squamous cell carcinomas (TSCCs).

MATERIAL AND METHODS: An immunohistochemical study was performed comprising a total of 42 tissue samples of patients suffering from TSCCs as well as 10 corresponding adjacent normal tissues. After detection of PRDX6 and CypA, their expression levels were semi-quantitatively evaluated and correlated with clinicopathologic variables.

RESULTS: Both PRDX6 and CypA expressions were significantly higher in tissue samples of TSCCs compared with the 10 corresponding adjacent normal tissues ($P < 0.01$). A statistically significant correlation in TSCCs regarding the expression of PRDX6 and CypA was revealed ($P = 0.005$), and the lymphadenectasis was correlated with PRDX6 ($P < 0.05$). Results of a multivariate analysis revealed age, CypA expression, cervical lymph node metastases, and tongue cancer differentiation to be independent prognostic variables in respect of the overall survival rate ($P < 0.05$).

CONCLUSIONS: It could be detected that PRDX6 and CypA are associated with tumorigenesis in TSCCs. High levels of CypA expression may predict reduced survival time.

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Keywords: PRDX 6; CypA; tongue cancer; prognosis

Introduction

Tongue squamous cell carcinoma (TSCC) is among the most frequent carcinomas in the world, being famous for its invasive nature, lymph node metastasis and poor prognosis. Its tumorigenesis and progression are closely linked to a hypoxic environment, which influences the outcome of chemo-, radiotherapy and surgery of squamous cell cancers of the head and neck region, as well as the survival rate of these patients (Zhang *et al*, 2004; Hoogsteen *et al*, 2007; Kim *et al*, 2007; Roh *et al*, 2009). Reactive oxygen species (ROS) plays an important role in the initiation, promotion and progression of cancer under hypoxic conditions, which has become of utmost interest in tumor research in recent years (Klaunig *et al*, 1998; Kusmartsev *et al*, 2004; Lim *et al*, 2010). The antioxidant and cell redox state modulating enzymes such as superoxide dismutases, glutathione peroxidases, catalase and peroxiredoxins (PRDXs) are increased to inhibit ROS and protect the non-tumor cells as well as tumor cells from apoptosis.

The thiol-specific antioxidant protein PRDX6, a novel family peroxiredoxins, eliminates H_2O_2 in cells. Recently, overexpression of PRDX6 has been reported in various tumors, including human renal cancer (Klaunig *et al*, 1998), lung cancer (Lehtonen *et al*, 2004), breast cancer (Li *et al*, 2006a), and pilocytic astrocytomas (Nordfors *et al*, 2007). So far, its role within human tumors has not yet been elucidated, even though the PRDXs are linked to cell proliferation, apoptosis, differentiation and gene expression in cancer. Moreover, the real electron donor of PRDX6 has not yet been clarified: different authors consider it to be cyclophilin A (CypA), glutathione, lipoid acid (Wood *et al*, 2003), reduced-glutathione (GSH) (Manevich *et al*, 2004) as well as glutathione S-transferase π (GSTπ) (Ralat *et al*, 2006). Cyclophilin A is a protein with a peptidylprolyl cis–trans-isom-erase (PPIse) activity, which, if binding to PRDX6, enhances the antioxidant activity of the latter (Lee *et al*, 2001). Cyclophilin A, being first identified as an intracellular receptor for cyclosporine A (CsA), (Handschumacher *et al*, 1984) has recently been detected to be associated with tumor pathogenesis, showing increased levels in various cancers including pancreatic adenocarcinoma, hepatocellular carcinoma, non-small cell lung cancer, and buccal

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squamous cell carcinoma (Yang *et al*, 2007). Furthermore, it was detected that CypA also played an important role within the tumorigenesis of solid tumors, and that increased levels protected cancer cells against hypoxia and cisplatin induced apoptosis, the latter being associated with suppressed ROS (Choi *et al*, 2007).

However, the molecular mechanisms between oxidant stress and the cellular redox-regulating enzymes in carcinomas have not yet been fully understood, as well as the contradictory reported significance of ROS and antioxidant systems within the carcinogenesis. The purpose of this preliminary study was to investigate the expression and relation of PRDX6 and CypA in TSCC, as well as their eventual influence on clinicopathologic features and outcomes.

Materials and methods

Patients and clinical data

The study comprised the specimens of 42 patients (26 men; 16 women; age range: 25–82 years, mean age: 51 years) suffering from TSCC. All of them have been treated surgically from 2002 to 2004 in the Department of Oral and Maxillofacial Surgery at the School of Stomatology at Wuhan University. The subsequent examination of their specimens has been performed at the Department of Oral Pathology. The histopathologic diagnoses were confirmed by two independent pathologists before immunostaining was performed. The clinical characteristics of these patients were obtained from the medical records, and are shown in Table 1. The age, gender, T category and lymphadenectasis were included in the study, the clinical stages of the TSCC were classified according to the guidelines of the International Union against Cancer (UICC 2002), the early clinical stage (clinical stage I–II) and the advanced clinical stage (clinical stage III–IV) were grouped. The grading scheme of the World Health Organization was used to determine the histologic grading (Barnes *et al*, 2005) as well differentiated, moderately differentiated, or poorly differentiated carcinoma. In addition, Wei *et al* (2009) thought that the sites at least 2 cm away from the edge of tumor mass could avoid contamination by the tumor cells, and 10 normal tissues adjacent to the cancerous lesion, which were collected at sites more than 2 cm from the edge of tumor mass, were used as a control group. The specimens were fixed within a 10% neutral buffered formalin solution and thereafter embedded in paraffin. Four μm thick sections were cut and mounted on precoated slides (Approved by Wuhan University Medical Ethics Committee). All cancer patients underwent a follow-up ranging from 2 to 75 months (mean: 48 months).

Immunohistochemical staining

All sections were incubated in a 0.01 M citric acid buffer solution (pH 6.0) for 1.5 min around the boiling point for antigen retrieval,then incubated in 3% hydrogen peroxide for 20 min, and treated with a working dilution of 10% normal goat serum for 30 min. Thereafter, they were incubated at 4°C overnight within a 1:3000 diluted solution of rabbit polyclonal anti-PRDX6 antibody (Abcam Biotechnology Inc, Cambridge, UK) as primary antibody solution. The detection of CypA staining was performed with a 1:100 dilution of rabbit polyclonal anti-CypA antibody (Proteintech Group Inc, Chicago, USA) Overnight, after the sections were put into the incubator at 37°C for 1 h, they were incubated within a second biotinylated goat antirabbit immunoglobulin G antibody (Santa Cruz, CA, USA) solution for 20 min at room temperature. Subsequently, they were treated with an avidin-biotin- peroxidase reagent for another 20 min. Development of the slides in diaminobenzidine (DAB), as well as a counterstaining with hematoxylin, resulted in the visualization of the immunostaining. Negative controls were treated in the same product but omitting the primary antibodies.

Table 1 Clinicopathologic parameters of 42 cases of tongue cancer

Variables	*Category*	n *(%)*
Age	< 60 years	31(73.8)
	≥60 years	11(26.2)
Gender	Male	26(61.9)
	Female	16(38.1)
T category	T1 + 2	34(81.0)
	T3 + 4	8(19.0)
Lymphadenectasis	No	34(81.0)
	Yes	8(19.0)
TNM stage	T1 + T2	23(54.8)
	T3 + T4	19(45.2)
Differentiation	Well	6(14.3)
	Moderate	31(73.8)
	Poor	5(11.9)
Lymphatic metastasis	No	28(66.7)
	Yes	14(33.3)
Local recurrence	Yes	13(31.0)
	No	29(69.0)
Distance metastasis	No	36(85.7)
	Yes	6(14.3)
Status	Alive	27(64.3)
	Dead	11(26.2)
	Missing	4(9.5)

Evaluation of the immunohistochemical results

Both scoring and interpretation of the immunostaining results were blindly interpreted by two independent oral pathologists under the light microscope at a magnification of ×200. According to the method by Kinnuna and Lehtonen *et al* (Kinnula *et al*, 2002; Nordfors *et al*, 2007), the immunostaining results were evaluated. A semiquantitative scoring system was used to determine both the percentage and the intensity of positive staining cells in cytosonic. The percentage of positive immunostained cells was graded and scored as follows: 0 = no stained cells; 1 = 1–25% positive cells; 2 = 26–50% positive cells; 3 = 51–75% positive cells; and 4 = more than 75% positive cells. Based on the intensity of positive reaction to PRDX6 and CypA of cancer cells, immunostaining was evaluated and scored as: 0 = absence (−), 1 = weak (+), 2 = moderate (++), or 3 = strong (+++). Subsequently, the overall score for each test specimen was divided into three groups by adding the extent score to the intensity score: negative (low) immunostaining (0–3), moderate immunostaining (4–5), and strong immunostaining (6–7).

Statistical analysis

Statistical data analysis was performed with SPSS software, version 16.0 (SPSS Inc., Chicago, IL, USA). The analysis of the correlation between PRDX6, CypA and clinicopathologic features was performed using the chi-squared test and/or Fisher's exact probability test, when appropriate. The Spearman rank correlation coefficient test and linear tendency test were applied for the correlation between PRDX6 and CypA. In all cases, the overall survival was defined as the time between the dates of surgery until the dates of last follow-up or death. Multivariate analysis was executed by means of the Cox proportional hazards regression analysis, which was performed by enter procedure. Differences were considered significant when the *P*-value was < 0.05.

Results

The levels of PRDX6 and CypA were evaluated by means of immunohistochemical analyses. An increased expression of PRDX6 and CypA was detected in most cases, although at different levels. However, negative immunostaining related with anti-PRDX6 antibody was found in corresponding adjacent normal specimens (Figure 1a). Low levels of PRDX6 were present in 28.6% (12/42) of TSCC specimens, moderate levels in 54.7% (23/42) specimens and strong levels in 16.7% (7/42) specimens. Positive PRDX6 staining was mainly localized in the cytoplasm of most cancer cells (Figure 1b,c). In the corresponding adjacent normal specimens, the CypA immunostaining was negative in cytoplasm, even though a scarce positive immunostaining was detected within the nuclei of prickle cells in a few specimens (Figure 1d). Low levels of CypA were present in 11.9% (5/42) TSCC specimens, moderate levels in 40.5% (23/42) specimens, strong levels in 47.6% (20/42) specimens. Moderate and strong immunostaining of CypA was evident within the cytoplasm and in the nucleus, diffuse in the latter (Figure 1e,f). The difference between PRDX6 and CypA immunostaining in tumor specimens and the corresponding adjacent normal specimens was significant ($P < 0.01$, Table 2).

Table 2 The differences in PRDX6 and CypA expression between tongue cancer and corresponding adjacent normal tissues

		Score		
	n	*Low (0–3)*	*Moderate & High (4–7)*	P
Cytosolic PRDX6				
Tongue cancer	42	12	30	0.001*
Adjacent normal tissues	10	9	1	
Cytosolic CypA				
Tongue cancer	42	5	37	0.000*
Adjacent normal tissues	10	8	2	

CypA, cyclophilin A.
*Fisher's exact test, $P < 0.01$.

To evaluate the relationship of PRDX6 expression and clinical features, the cross-tabs chi-square test was performed. Table 3 highlights that whereas age, gender, cancer diameter, clinical stage and the degree of cell differentiation, recurrence and distant metastasis ($P > 0.05$) are not, the cervical lymph node size is associated with an increased immunoexpression of PRDX6 ($P = 0.016$). Similarly, the correlation between clinicopathologic features and the CypA expression was investigated; no clinicopathologic feature was significantly related to the CypA expression ($P > 0.05$). Figure 2 shows a highly significant association between PRDX6 and CypA expression ($P = 0.005$) in TSCCs.

The month was set as unit of the minimum follow-up time after treatment, all patients underwent a follow-up ranging from 2 to 75 months at the deadline of December 2008, and the short follow-up time was due to patient death. The mean follow-up period was 48.21 ± 23.85 months and the median time was 57 months. At the end of study of 42 patients, 4 patients were lost during follow-up, 27 patients were alive, 13 patients suffered from a recurrence, 5 patients showed pulmonary metastases, 1 patient had lung and bone metastases,11 had died of cancer, the 5-year overall and disease-free survival rates were 71.1% and 65.8%, respectively. A multivariate analysis (Cox's proportional hazards model Table 4) revealed that

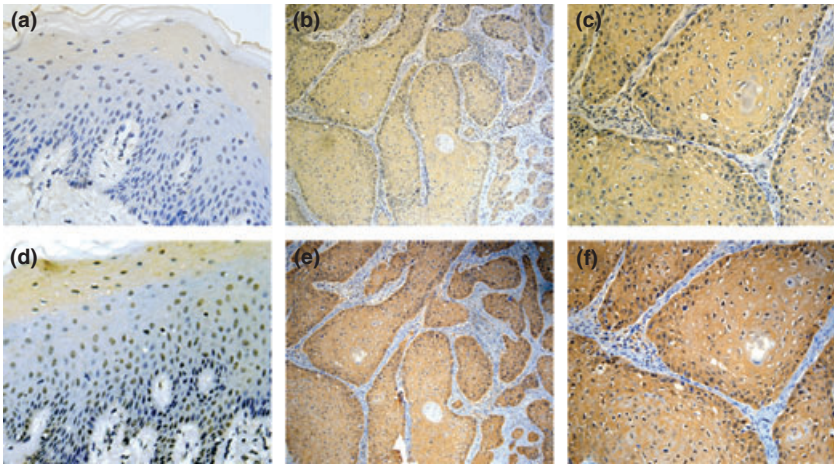

Figure 1 Immunohistochemical expression of PRDX6 in corresponding adjacent normal tissue (**a**) and tongue squamous cell carcinoma (**b, c**); CypA expressed in corresponding adjacent normal tissue (**d**) and tongue squamous cell carcinoma (**e, f**). (**a, d**: original magnification ×400; **b, e**: original magnification ×100; **c, f**: original magnification ×200)

Table 3 Correlation with PRDX6 and CypA expression and clinicopathologic features

		Cytosolic PRDX6				*Cytosolic CypA*			
Clinicopathologic features	n%	*0–3*	*4–5*	*6–7*	P	*0–3*	*4–5*	*6–7*	P
Age									
< 60	31 (73.8)	10	17	4	0.456	4	14	13	0.462
≥60	11 (26.2)	2	6	3		1	3	7	
Gender									
M	26 (61.9)	7	13	6	0.362	4	10	12	0.673
F	16 (38.1)	5	10	1		1	7	8	
T category									
T1 + 2	34 (81.0)	10	19	5	0.780	5	14	15	0.437
T3 + 4	8 (19.0)	2	4	2		0	3	5	
Lymphadenectasis									
No	34 (81.0)	10	21	3	0.016*	3	15	16	0.364
Yes	8 (19.0)	2	2	4		2	2	4	
TNM stage									
T1 + T2	23 (54.8)	4	16	3	0.097	1	9	13	0.191
T3 + T4	19 (45.2)	8	7	4		4	8	7	
Differentiatio									
Well	6 (14.3)	3	3	0	0.376	1	3	2	0.592
Moderate	31 (73.8)	9	16	6		4	13	14	
Poor	5 (11.9)	0	4	1		0	1	4	
Lymphatic metastasis									
No	28 (66.7)	9	15	4	0.711	3	12	13	0.886
Yes	14 (33.3)	3	8	3		2	5	7	
Local recurrence									
Yes	13 (31.0)	4	7	2	0.974	2	7	4	0.342
No	29 (69.0)	8	16	5		3	10	16	
Distance metastasis									
No	36 (85.7)	11	19	6	0.768	5	13	18	0.314
Yes	6 (14.3)	1	4	1		0	4	2	

CypA, cyclophilin A
*$P < 0.05$ by Cross-tabs chi-square test.

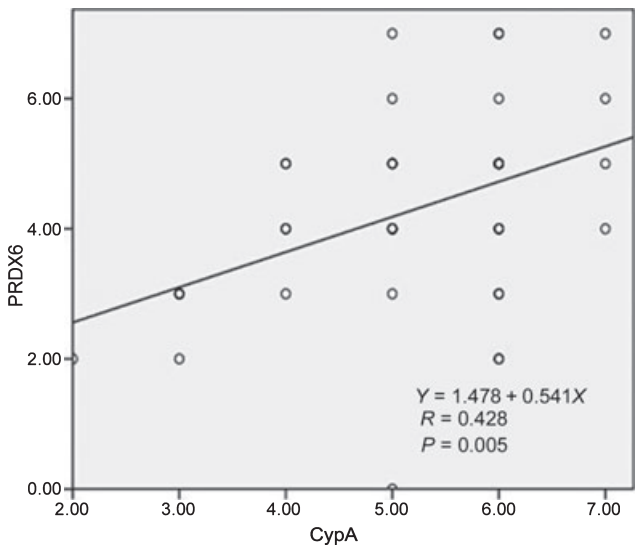

Figure 2 Correlation between the expressions of PRDX6 and cyclophilin A (CypA). Spearman correlation and linear regression were used to determine the relationship between PRDX6 and CypA. There were significant correlation between PRDX6 and CypA at $P = 0.005$

age, CypA, cervical lymph node metastasis and tongue cancer cell differentiation had an independent prognostic effect on the overall survival rate ($P < 0.05$).

Table 4 Prognostic factors by Multivariate analysis (Cox's proportion hazards model)

Variables	*Relative Risk (95% confidence interval)*	P
Age (< 60/ > 60)	0.858 (0.740–0.997)	0.046*
PRDX6 (0–3/4–7)	1.565 (0.189–12.952)	0.678
CypA (0–3/4–7)	0.036(0.002–0.648)	0.024*
Gender (M/F)	47.168 (0.785–2.853)	0.065
T category (T1 + 2/T3 + 4)	33.756 (0.313–3.641)	0.141
Lymphadenectasis (N/Y)	0.003 (0.000–2.353)	0.087
Node metastasis(N/Y)	0.024 (0.001–0.771)	0.035*
Differentiation (G1/G2 + G3)	93.045 (2.350–3.684)	0.016*
TNM stage (I + II/III + IV)	99.517 (0.061–1.628)	0.223
Recurrence (N/Y)	9.695 (0.459–204.790)	0.144
Distant metastasis (N/Y)	7.755 (0.562–107.080)	0.126

N, no; Y, yes, M, male; F, female; *P < 0.05 by multivariate Cox proportional hazard model.

Discussion

This study investigated the expression of the antioxidant protein PRDX6 and CypA in TSCC. In lung cancer PRDX6 was highly expressed in nuclear but scarcely in cytoplasm (Lehtonen *et al*, 2004), and it was over expressed in both nuclear and cytoplasm in malignant mesothelioma (Kinnula *et al*, 2002). In pilocytic astrocytomas, PRDX6 expression was strong in 45%

cases, moderate in 37% cases, and weak in 15% cases (Handschumacher *et al*, 1984). The proportion of strong immunostaining of PRDX6 was 16.7%, moderate 54.7% and low 28.6% in our study, and the corresponding adjacent normal tissues showed mostly negative and weak expression of PRDX6. CypA was mainly expressed in cytoplasm, however, also evident within nuclei in a few cases, in the corresponding adjacent normal tissues. CypA was very scarcely expressed within nuclei of some prickle cells, which is in accordance with the findings in pancreatic cancer (Li *et al*, 2006b) and endometrial carcinoma (Li *et al*, 2008), adjacent normal pancreatic tissue composed of acinar cells and ductal epithelial cells with very weak immunostaining of CypA, and normal endometrium showed negative CypA immunostaining in cytoplasm and weakly positive in nuclei. In addition, there was no CypA immunostaining in adjacent normal lung tissue (Campa *et al*, 2003).

PRDX6 is an important antioxidant enzyme in cells. It regulates the cellular redox state also in malignant cells, with potential effects on tumor growth and apoptosis. According to the research of Nordfors *et al* (2007), it is known that the proteins of the PRDX family are strongly expressed in pilocytic astrocytomas, indicating that oxidative damage and consequent defense takes place during tumor's progression. Accumulation of ROS, might affect any cellular macromolecule at least during the carcinoma initiation stage, especially in hypoxic environment (Evans *et al*, 2004). Moreover, antioxidant enzymes such as PRDX6 will also increase to protect normal cells and inhibit ROS-mediated physiologic apoptosis, leading probably to abnormal proliferation and carcinogenesis (Chang *et al*, 2007). The specific molecular mechanisms of how ROS and PRDX6 influence the carcinogenesis, however, remain sophisticated and unexplained to date. On the one hand, PRDX6 is an antioxidant agent and protects the organism from ROS-induced tumorigenesis by scavenging ROS; on the other hand, it protects the cells from oxidative stress-induced apoptosis, inducing tumorigenesisNeumann and Fang (2007) hypothesized that PRDXs are over expressed in various cancer cells, protecting cancer cells by elimination of ROS-induced apoptosis through their peroxidase activity. CypA has been recently described to bind to PRDX6, which protects cells from oxidative stress-induced apoptosis, being therefore as well correlated with carcinogenesis. In buccal squamous cell carcinomas, CypA was identified by proteomic technology to be a tumor-associated protein that participates in intracellular signaling pathways of buccal tumorigenesis, inhibiting T-cell receptor-mediated signal transductions, regulating therefore the T cell activation (Chen *et al*, 2004). Campa *et al* (Campa *et al*, 2003) considered CypA to be an important molecule in tumorigenesis due to its multifunctional nature including, for example, the cellular growth and differentiation, the transcriptional control, cell signaling pathways and the immunosuppression.

This study showed an increased expression of PRDX6 and CypA in TSCC. The decreased level of ROS due to the interaction of PRDX6 and CypA in TSCC might lead to cell proliferation. The finding that CypA alone also displayed the ability to eliminate ROS and simulated cell proliferation, nurtures speculations that the compound of CypA and PRDX6 might increase the effects on ROS and consecutively on tumorigenesis. However, the molecular mechanisms among ROS, PRDX6 and CypA are complex and partly contradictory. In future, further investigations will be needed.

Cancer invasiveness usually is combined with a poorer survival rate. In breast cancer, it was shown that the PRDX6 expression was significantly associated with metastasis (Li *et al*, 2006a). And inhibition of PRDX6 expression in breast cancer cells resulted in decreased cancer cell proliferation and invasion as well as in less pulmonary metastases (Chang *et al*, 2007). Recently, it has been reported that CypA stimulated cancer cell proliferation by interaction with its cellular receptor CD147 via extracellular signal-regulated kinases Erk1/2 and p38 mitogen-activated protein kinases (MAPKs) (Li *et al*, 2006b). In this study, expression of PRDX6 was correlated with lymphadenectasis, but not lymph node metastases, local recurrence and distant metastases in TSCC, perhaps the level of PRDX6 protein was increased following the reactive hyperplasia of lymph node in the carcinogenesis and progression of the tongue cancer, and the specific mechanism needs further research studies.

Furthermore, autoantibody response has been considered a new way to detect early-stage malignant tumor and establish effective immunotherapies (Nesterova *et al*, 2006; Shoshan and Admon, 2007). The autoantibody against PRDX6 was found in esophageal squamous cell carcinoma sera, which could be a novel serum marker with esophageal squamous cell carcinoma (Fujita *et al*, 2006). Both PRDX6 and CypA were antigens, which elicit an autoantibody response in cancer of gingivo-buccal complex (Shukla *et al*, 2007), but the autoantibody response to the antigens was very minimal in tongue cancer than gingivo-buccal complex cancer, may be their clinicopathologic features and biological origin were different (Shukla *et al*, 2009). In a recent study (Nordfors *et al*, 2007), PRDX6 was related to a significantly improved recurrence-free survival time. It, however, has not been associated with disease-free survival time in human bladder cancer(Quan *et al*, 2006). The data of this study did not show significant correlations between PRDX6 expression and the survival time, as shown by the results of Lehtonen *et al* (2004) in lung cancer. However, the authors presume that PRDX6 might be a potential prognostic factor, especially due to its influence upon the tumorigenesis and the progression of tumors. However, in endometrial cancer, over expression of CypA was reported to be correlated with a decreased survival time, being therefore considered a novel prognostic factor as well as an adequate treatment target (Li *et al*, 2008). This study detected age, CypA, cervical lymph node metastasis and tongue cancer cell differentiation were independent prognostic factors in TSCC, indicating that CypA might be a novel independent prognostic factor in TSCC.

In conclusion, this study demonstrated molecular mechanisms of PRDX6 and CypA in TSCC. A statistically significant association between PRDX6 and CypA over-expression was detected, probably representing a potentiating effect within the tumorigenesis. Moreover, it was revealed that CypA is considered an independent prognosis factor. Both proteins might be contemplated to be biomarkers for the prediction as well as potential targets for molecular treatment strategies in cancers. Future research, however, is needed to clarify further functions and interactions of PRDX6 and CypA.

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Conflict of Interest

The authors declare no competing financial interest.

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