The overexpression of PRDX4 modulates the tumor microenvironment and promotes urethane-induced lung tumorigenesis

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Abstract:

Background: Peroxiredoxin 4 (PRDX4), initially reported as an antioxidant, is overexpressed in lung cancer and participates in its progression. However, its role in urethane-induced lung tumor models is undetermined. We investigated the effect of the overexpression of PRDX4 on carcinogen-induced lung tumor development.

Materials and methods:

Human PRDX4 overexpression transgenic (Tg) mice (*hPRDX4*$^{+/+}$) and non-Tg mice were intraperitoneally injected with urethane to induce lung tumor development. After six months, their tumor formation was compared and possible mechanisms of the difference in tumor development were investigated.

Results:

The serum and lung PRDX4 expression were enhanced after urethane stimulation in Tg mice. Both the average number of tumors (≥0.5 mm) and tumor diameter per mouse in the Tg group were significantly larger than in non-Tg controls, while body weight was lower in the Tg group. The Tg group showed enhanced tumor cell proliferation and suppressed tumor cell apoptosis; systemic oxidative stress and oxidative stress in the lung tumors were inhibited by the overexpression of PRDX4. In lung tumor tissue, the density of microvessel penetration into the tumor was higher in the Tg group; macrophage infiltration was enhanced in Tg tumors, while there was no difference in T lymphocyte infiltration; the expression of cytokines, including interleukin 1 beta (IL-1β) and matrix metallopeptidase 9 (MMP9) were elevated in Tg tumors, which resulted from enhanced phosphorylation of nuclear factor-κB p65

(NF-κB p65) and c-jun respectively.

Conclusions:

The overexpression of PRDX4 modulated the tumor microenvironment and promoted tumor development in the mouse urethane-induced lung cancer model.

Keywords: PRDX4, lung tumorigenesis, tumor microenvironment, oxidative stress.

Background

Peroxiredoxin 4 (PRDX4) is ubiquitously expressed in mammalian cells (1) and is the only secretary member of the anti-oxidant peroxiredoxin family (2, 3). With typical 2-cysterine residuals, the most profound function of PRDX4 in cells is to suppress oxidative stress by eliminating H_2O_2. Given that oxidative stress can activate inflammation, lead to tumor transformation, and modulate tumor progression (4), the involvement of PRDX4 in tumors has been extensively studied. The increased expression of PRDX4 has been observed in many cancers, including prostate cancer (5), glioblastoma (6), oral cavity squamous cell carcinoma (7), and ovarian cancer (8), whereas the decreased expression of PRDX4 in tumors was only reported in very few cancers, for example, in acute promyelocytic leukemia (9) and gastric adenocarcinoma (10). Although many studies have shown that PRDX4 promotes tumor progression, such as the enhancement of invasion or metastasis (7, 11-15) and the augmentation of proliferation (5, 6, 15, 16), the role of PRDX4 in tumors is complicated and specific in certain tumor types. In our previous study in hepatocellular carcinoma (HCC), we found that PRDX4 inhibits the initiation of HCC but plays a complex role in tumor progression (17).The latest study in hepatoblastoma (HB) indicated that PRDX4 promoted embryonal hepatoblastoma cell migration but induced fetal hepatoblastoma cell differentiation (18). As different levels of reactive oxygen species (ROS), including H_2O_2, in the tumor microenvironment exert specific effects on the tumor (19), the tumor microenvironment may also affect the role of PRDX4 in the tumor via crosstalk between PRDX4 and oxidative stress. In summary,

PRDX4 plays different roles in different tumor contexts, including the tumor histological type, tumor stage, and even the tumor microenvironment, such as the oxidative stress balance.

Lung cancer ranks is the leading cause of cancer-related deaths worldwide (20). The majority of lung cancer patients are pathologically diagnosed with non-small-cell lung cancer (NSCLC) (21), which is associated with a poor prognosis. An early diagnosis is crucial for improving the prognosis. The identification of new diagnostic markers and novel therapeutic targets can help to restrain this malignant tumor. As a protein closely related to cancer, PRDX4 has been found to be overexpressed in lung cancer, especially adenocarcinoma (22-24). *In vitro*, the knockdown of PRDX4 in the A549 lung cancer cell line results in the formation of fewer colonies and reduced Matrigel invasion (25), whereas the overexpression of PRDX4 enhances anchorage-independent colony formation and Matrigel invasion (26). *In vivo*, PRDX4-positive staining was correlated with increased rates of recurrence and reduced disease-free survival (DFS) in squamous cell carcinoma patients (23), while our recent studies in stage I lung adenocarcinoma found that the weak expression of PRDX4 combined with a high MIB-1 labelling index predicts shortened DFS (27) and the high expression of PRDX4 combined with EGFR mutation was positively correlated with a better prognosis (28). In comparison to massive studies on lung cancer progression, the role of PRDX4in lung cancer development is still undetermined. Theoretically, PRDX4 can reduce oxidative stress and prevent lung carcinogenesis, but PRDX4 can be secreted into extracellular space and extracellular

PRDXs have been demonstrated to be capable of activating the expression of inflammatory cytokines and initiating post-ischemic inflammation in the brain (29). Secreted or extracellular PRDX4 may activate inflammation, which can lead to oncogenesis, like its family member PRDX1 (30). It has been proven that PRDX6, another PRDX family member, promotes the development of urethane-induced lung adenocarcinoma in mice (31).

As a carcinogen, urethane has been widely used to induce pulmonary adenoma in mice, which mimics human lung adenocarcinoma and offers important insights in tumor development (32). In the present study, we examined the effect of the overexpression of PRDX4 on the development of lung adenoma induced by urethane by comparing tumor formation between PRDX4 transgenic (Tg) mice and non-Tg mice.

Materials and methods

Ethics statement

All animal experiments were approved by the Ethics Committee of Animal Care and Experimentation, Kanazawa Medical University, Japan, and were carried out according to institutional guidelines for animal experiments and the law (no. 105) and notification (no. 6) of the Japanese government. Isoflurane was used as a euthanasia agent in the animal experiments.

Animal experiments

The construction of hPRDX4 Tg mice on the C57BL/6 background was detailed in

our previous study (33). C57BL/6 mice were purchased from Charles River Laboratories (Yokohama, Japan) as a non-Tg control. All mice were housed and bred under specific-pathogen-free conditions at the Animal Research Center of Kanazawa Medical University. All mice were kept in a room with relatively constant temperature (21-23℃), humidity (50-60%), and 12-hour light/dark circle. They were fed with standard chow with *ad libitum* access to purified tap water. PRDX4 Tg and non-Tg male mice (8 weeks old, n=15 for each group) were subjected to intraperitoneal injection of urethane (1g/kg in 100 ul saline) once per week for 16 consecutive weeks. Body weights were recorded weekly. Mice were sacrificed at 6 months after the initial injection. The lungs were harvested and the number of lung tumors and their diameter on the lung surface were measured. Serum was also collected after centrifugation. Another group of mice (8 weeks old, n=3 for each group) received one urethane injection every other day and were sacrificed after one week (short-term urethane stimulation trial).

Histology and immunohistochemistry

Harvested lungs were immersed in 4% paraformaldehyde for over 24 hours and then embedded in paraffin. Sections (thickness: 3-5 μm) were subjected to hematoxylin and eosin (H&E) and immunohistochemical (IHC) staining. For IHC staining, the procedure was as follows: 1) deparaffinization and rehydration; 2) 0.5% hydrogen peroxide blocking for 15 minutes at room temperature; 3) antigen retrieval: heat –mediated antigen retrieval (trypsin-mediated antigen retrieval was exclusively applied for PRDX4); 4) 3% bovine serum albumin blocking for 30 minutes at room

temperature; 5) primary antibody incubation overnight at 4℃; 6) Secondary antibody (Histofine Simple Stain MAX-PO424152) staining for 30 minutes at room temperature; and 7) 3, 3’ diaminobenzidine (DAB) imaging and hematoxylin counterstaining. H&E and IHC staining images were captured and quantitatively analyzed using the NanoZoomer Digital Pathology Virtual SlideViewer software program (Hamamatsu Photonics Corp., Hamamatsu, Japan).

PRDX4 (Human) ELISA assay

Blood was drained from the mouse axillary vein and then centrifuged. Serum was obtained and diluted 20-fold for the detection of hPRDX4. A PRDX4 (Human) ELISA kit (Abnova, KA2121) was used for this analysis. The absorbance at 450 nm was detected and the final serum hPRDX4 concentration was calculated.

Mouse whole-transcript array

A GeneChip® Mouse Gene 2.0 ST Array (Affymetrix, Inc.) was used for the mouse transcription analysis. Tumors were first resected from lung tissue, then RNA was extracted. After examination of the RNA quality, the whole-transcript array analysis was performed in a step-by-step manner according to the manufacturer’s instructions. Three mice per group were included in this analysis. Data were analyzed by an Ingenuity® pathway analysis.

Real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from lung tumor tissue using a ReliaPrep™ RNA TissueMiniprep System (Promega Corporation, USA) and was stored at -80°C. Conversion to cDNA was conducted by a High Capacity RNA-to-cDNA kit (Life

Technologies). cDNA was amplified (40 cycles) in Applied Biosystems™QuantStudio™12K FlexReal-Time PCR System, with the help of TaqMan gene expression assays (Life Technologies). Each sample was tested in triplicate for the target gene. *18S* was used as the reference gene. The comparative Ct method was used for the data analysis. Custom-produced primers and TaqMan probes for amplification of the target genes were purchased from Life Technologies (Assay ID: Hs01056076_m1).

Western-blotting (WB)

Lung tumor tissue was homogenized in RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific) to which protease inhibitor had been added. 10-50 ug protein extract was loaded onto 5-12.5% SDS-PAGE gels (Bio-Rad) and transferred to nitrocellulose membranes (Bio-Rad) after electrophoresis. Membranes were blocked in 3% bovine serum albumin (BSA) and then probed with corresponding antibodies. The ImageJ software program was used for band quantification.

Thiobarbituric Acid Reactive Substances (TBARS) assay

A TBARS Assay Kit (Cayman Chemical, USA) was used for the quantification of malondialdehyde (MDA) in mouse serum. The detailed protocol is listed in the product datasheet.

Antibodies used in IHC and WB

PRDX4 (PA3-753, Thermo Fisher, 1:1000 in IHC and WB), PCNA (sc56, Santa Cruze Biotechnology, 1:200 in IHC and 1:1000 in WB), 8-OHdG (N45.1, Japan Institute for the Control of Aging; 1:200 in IHC), Mac-2 (CL8942LE, Cedarlane

Laboratories, 1:1000 in IHC), CD3 (ab5690, Abcam, 1:100 in IHC), CD31 (DIA-310, Dianova, 1:20 in IHC), Cleaved Caspase-3 (# 9661S, Cell Signaling Technology, 1:200 in IHC and 1:1000 in WB), IL-1β(# 12242S, Cell Signaling Technology, 1:1000 in WB), NF-κBp65 (# 8242S, Cell Signaling Technology, 1:1000 in WB), Phospho-NF-κB p65 (#3033S, Cell Signaling Technology, 1:1000 in WB), TNF-α(# 3707S, Cell Signaling Technology, 1:1000 in WB), Phospho-c-Jun (Ser73)(#3270, Cell Signaling Technology, 1:1000 in WB), c-Jun (#9165, Cell Signaling Technology, 1:1000 in WB), MMP3 (ab52915, 1;1000 in WB), MMP9 (ab38898, Abcam, 1:1000 in WB).

Statistical analysis

Variables were expressed as the mean±SD. The two-tailed independent student's t-test was used for comparisons. All statistical analyses were performed using the SPSS statistical software package, version 16.0. Two-sided *P* values of <0.05 were considered to indicate statistical significance.

Results

The elevated expression of PRDX4 in Tg mice after urethane stimulation

Before urethane injection, no significant the expression of PRDX4 difference was found in lungs between Tg mice and non-Tg mice. We also found no difference in the expression of PRDX4 between lung tumors from these two groups. However, lung the expression of PRDX4 was significantly higher in Tg mice than in non-Tg controls after short-term urethane stimulation (one injection every other day for one week)

(**Figure 1A**). An intermediate level of serum hPRDX4 was found in Tg mice before urethane injection (29.93±2.50 ng/ml). The level was increased after short-term urethane stimulation (44.82±10.22 ng/ml) and decreased after tumor formation (11.51±2.62 ng/ml), which may be explained by senescence and cancer-associated cachexia. No or very weak serum hPRDX4 was detected in non-Tg mice (**Figure 1B**).

Promotion of tumor development in PRDX4 Tg mice

The number of tumors (≥0.5mm) and their diameter on the lung surface were measured after mice were sacrificed. The average number of tumors per mouse in PRDX4 Tg mice was significantly greater in comparison to non-Tg controls (Tg 11.00±3.06 vs. non-Tg 6.73±1.85, $p<0.001$). Moreover, the average tumor diameter (mm) in the Tg group was also larger than in non-Tg controls (Tg 1.30±0.17 vs. non-Tg 0.96±0.14, $p<0.01$) (**Figure 2A**). H&E staining further confirmed this finding, with increased tumor formation in Tg mice (**Figure 2B**). In accordance with increased tumor formation, the average body weight (g) of Tg mice was significantly smaller than in non-Tg controls at the time of sacrifice (Tg 26.88±2.23 vs. non-Tg 29.64±2.22, $p<0.05$) (**Figure 2C**).

Tumor apoptosis and enhanced tumor proliferation were suppressed in Tg mice. Immunohistochemical staining of cleaved caspase-3 revealed that fewer cells underwent apoptosis in Tg tumors (Tg 11.40±2.70 vs. non-Tg 23.80±3.03, $p<0.001$). Western blotting also demonstrated that the expression of cleaved caspase-3 was significantly weaker in Tg tumors than in non-Tg control tumors, while the PCNA expression in Tg tumors was stronger than non-Tg control tumors (**Figure 3**).

Increased microvascular permeability and macrophage infiltration in lung tumors of PRDX4 Tg mice

In IHC staining, the number of microvessels (per one high power field), as demonstrated by CD31-positive cells in the lung tumors of Tg mice, was significantly greater in comparison to non-Tg mice (Tg 12.50±2.08 vs. non-Tg 2.00±0.82, $p<0.001$), indicating increased microvascular permeability. CD3 lymphocytes were mainly found in the marginal area of the tumor and there was no significant difference between two groups. Macrophage (Mac-2-positive cells) infiltrated into the tumor and were more frequently detected in Tg tumors than in non-Tg controls (Tg 3.83%±0.40% vs. non-Tg 1.76%±0.07%, $p<0.001$) (**Figure 4**).

The elevated expression of MMP9 and IL-1β in Tg tumors

A mouse whole-transcript array revealed that there were significant differences in 17 transcripts between groups when a 1.5-fold change was used as the threshold. Only 4 transcripts (*Hspa1b*|*Hspa1a*, *Igkv12-44*, *Igkv3-5*, and *Igkv4-91*) showed 2-fold change (**Supplementary Table 1**). RT-PCR revealed a >3-fold increase in the expression of *MMP9* and an over 2-fold increase in the expression of *MMP13* in Tg tumors in comparison to non-Tg controls. A cytokine expression analysis, including *IL-1β*, *IL-6*, *TNF-α*, and *iNOS* also showed a tendency toward increased expression in Tg tumors; however, the difference was not statistically significant (**Figure 5A**). WB showed the obvious elevation of the MMP9 and IL-1β expression in Tg mice, whereas no difference was found in TNF-α or MMP3. The phosphorylation of NF-κB p65 and c-jun were significantly enhanced in Tg tumor tissue, indicating activation of NF-κB

and activator protein 1 (AP-1) pathway (**Figure 5B**).

Inhibition of oxidative stress in Tg mice

IHC staining of 8-OHdG in tumors revealed that there were fewer positively stained cells in Tg tumors in comparison to non-Tg tumors (positively stained proportion: Tg 88.75%±3.00% vs. non-Tg 11.25%±1.50%, $p<0.001$), indicating obvious inhibition of local oxidative stress in Tg tumors (**Figure 6A**). A TBARS assay showed that the MDA concentration (uM) in Tg serum was significantly lower than in non-Tg controls after tumor formation (Tg 32.10±13.39 vs. non-Tg 42.60±11.58, $p<0.05$), indicating a decrease in systemic oxidative stress (**Figure 6B**).

Discussion

In the present study, we demonstrated the overexpression of PRDX4, the only secreted member of the PRDX family, promotes tumor development in urethane-induced lung adenoma. In our study, greater numbers of lung carcinoma tumors and larger tumors were found in PRDX4 Tg mice, accompanied by enhanced macrophage infiltration and the elevated expression of IL-1β and MMP9 in the tumor microenvironment. The tumor microenvironment is the surrounding environment where the tumor develops and survives. Its components include immune cells, cytokines and even products of oxidative stress. Each component is critical for tumorigenesis. It is widely accepted that macrophages participate in tumor initiation and progression (34-38). In the phase of oncogenesis, inflammatory cells, including macrophages, contribute to geneticmutations and instability (38). In the tumor

progression phase, tumor associated macrophages promote angiogenesis and remodel extracellular matrix (39-42). Previous studies on the role of macrophages in a urethane-induced mouse lung adenoma model verified that macrophages promote both cancer carcinogenesis (43) and progression (44). In our present study, more macrophages emerged in the tumor tissue, which may partly explain the effect of PRDX4 overexpression in the promotion of tumor development. Although no key players in lung cancer development were found to be significantly changed after the overexpression of PRDX4 in a whole-transcript array, other analyses (RT-PCR and WB) revealed some important positive findings. As a critical cytokine in inflammation, IL-1β has been demonstrated to efficiently promote chemical-induced carcinogenesis (45, 46). In this study, the expression of was obviously increased in the PRDX4 Tg tumor microenvironment. The elevation of IL-1β may also account for enhanced lung tumor development in Tg mice. The relationship between the overexpression of PRDX4 and AP-1 activation has been well elucidated in the A549 lung cancer cell line (25, 26). PRDX4 was critical for the activation of AP-1 signaling and the depletion of PRDX4 leads to the reduced phosphorylation of c-Jun and the decreased expression of MMP9, which contributes to the malignancy of human lung cancer cells. The present study verified a similar relationship in an animal model of carcinogen-induced lung cancer. With the overexpression of PRDX4 in mice, the increased phosphorylation of c-Jun (Ser 73) was observed, and the expression of MM9 was elevated in sequence. MMP9 was critical for tumor angiogenesis and extracellular matrix remodeling. In accordance with the elevated MMP9 expression,

the expression of CD31 was observed to be increased in PRDX4 Tg tumor tissue, indicating a higher density of microvessels, which benefits the development of lung adenoma in mice. All of the above findings indicated that a changing microenvironment, which favored tumor development, was created after the overexpression of PRDX4. One seemingly contradictory phenomenon in our study is that the 8-OHdG and MDA levels were decreased in Tg mice, which represents a lower oxidative stress level and theoretically leads to prevention of tumor initiation. However, the role of oxidative stress in cancer development are complicated and elevated oxidative stress potentially plays a uniquely dual (double-faced) role in the different (early to late) stages of lung tumorigenesis, including pro-tumorigenic and tumor suppressing effects. It is still very difficult to accurately define which level of oxidative stress is mild, moderate or severe, in these *in vivo* experiments and which level is the most beneficial in a specified tumor development. In our study, the relatively lower oxidative stress level in Tg mice may favor lung tumor development via the promotion of tumor proliferation and the inhibition of tumor apoptosis.

The balance of oxidative stress and antioxidants is crucial to maintaining our body health. An imbalance can lead to a pathological condition, including inflammation and cancer (4). Although antioxidant therapy has been demonstrated to be beneficial in inflammation and ischemia/reperfusion injury (47), certain antioxidants may exert a totally reverse effect. A study on stroke revealed that PRDXs were key initiators of post-ischemic inflammation (29). Another study found that the PRDXs released by

cancer cells can mediate osteoclastogenesis (14), a process mainly performed by macrophages. Our recent study demonstrated that the overexpression of PRDX4 was associated with the aggravation of inflammation in idiopathic pulmonary fibrosis (48). Briefly, antioxidant therapy should be approached with caution, even in inflammatory disease, and the treatment should be based on the administration of certain antioxidants for specified inflammation. Given the conflicting effect of antioxidant therapy in inflammation and the close relationship between tumors and inflammation, it is easy to understand the diverse roles of antioxidants in tumor development. Antioxidants like PRDX4 may suppress liver inflammation and prevent hepatocellular carcinoma, but may exacerbate pulmonary inflammation and promote the development of lung adenocarcinoma. Even in a certain types of tumor, antioxidant supplementation may have totally different effects according to different periods in the tumor, such as the initiation phase or progression phase, the early stage or the late stage. When performing antioxidant therapy, physicians should be especially cautious when considering aspects of the tumor context, such as the tumor histological classification as tumor stage, as well as aspects of the tumor microenvironment, such as oxidative stress.

In conclusion, the overexpression of PRDX4 promotes urethane-induced lung tumorigenesis, and the alteration of the microenvironment caused by the high expression of this antioxidant enzyme may play important roles in this process. The results of the present study could provide novel insights in relation to antioxidant

therapy for lung cancer.

References

1. Rhee SG, Chae HZ, Kim K. Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling. Free Radic Biol Med. 2005;38(12):1543-52.

2. Fujii J, Ikeda Y, Kurahashi T, Homma T. Physiological and pathological views of peroxiredoxin 4. Free Radic Biol Med. 2015;83:373-9.

3. Yamada S, Guo X. Peroxiredoxin 4 (PRDX4): Its critical in vivo roles in animal models of metabolic syndrome ranging from atherosclerosis to nonalcoholic fatty liver disease. Pathology International. 2018;68(2):91-101.

4. Reuter S, Gupta SC, Chaturvedi MM, Aggarwal BB. Oxidative stress, inflammation, and cancer: how are they linked? Free Radic Biol Med. 2010;49(11):1603-16.

5. Ummanni R, Barreto F, Venz S, Scharf C, Barett C, Mannsperger HA, et al. Peroxiredoxins 3 and 4 are overexpressed in prostate cancer tissue and affect the proliferation of prostate cancer cells in vitro. J Proteome Res. 2012;11(4):2452-66.

6. Kim TH, Song J, Alcantara Llaguno SR, Murnan E, Liyanarachchi S, Palanichamy K, et al. Suppression of peroxiredoxin 4 in glioblastoma cells increases apoptosis and reduces tumor growth. Plos One. 2012;7(8):e42818.

7. Chang K-P, Yu J-S, Chien K-Y, Lee C-W, Liang Y, Liao C-T, et al. Identification of PRDX4 and P4HA2 as metastasis-associated proteins in oral cavity squamous cell carcinoma by comparative tissue proteomics of microdissected specimens using iTRAQ technology. J Proteome Res. 2011;10(11):4935-47.

8. Pylvas M, Puistola U, Kauppila S, Soini Y, Karihtala P. Oxidative stress-induced antioxidant enzyme expression is an early phenomenon in ovarian carcinogenesis. Eur J Cancer. 2010;46(9):1661-7.

9. Palande KK, Beekman R, van der Meeren LE, Beverloo HB, Valk PJM, Touw IP. The antioxidant protein peroxiredoxin 4 is epigenetically down regulated in acute promyelocytic leukemia. Plos One. 2011;6(1):e16340.

10. Jang JS, Cho HY, Lee YJ, Ha WS, Kim HW. The differential proteome profile of stomach cancer: identification of the biomarker candidates. Oncol Res. 2004;14(10):491-9.

11. Basu A, Banerjee H, Rojas H, Martinez SR, Roy S, Jia Z, et al. Differential expression of peroxiredoxins in prostate cancer: consistent upregulation of PRDX3 and PRDX4. Prostate. 2011;71(7):755-65.

12. Song P, Bao H, Yu Y, Xue Y, Yun D, Zhang Y, et al. Comprehensive profiling of metastasis-related proteins in paired hepatocellular carcinoma cells with different metastasis potentials. Proteomics Clin Appl. 2009;3(7):841-52.

13. Li M, Lin Y-M, Hasegawa S, Shimokawa T, Murata K, Kameyama M, et al. Genes associated with liver metastasis of colon cancer, identified by genome-wide cDNA microarray. Int J Oncol. 2004;24(2):305-12.

14. Rafiei S, Tiedemann K, Tabaries S, Siegel PM, Komarova SV. Peroxiredoxin 4: a novel secreted mediator of cancer induced osteoclastogenesis. Cancer Lett. 2015;361(2):262-70.

15. Wang W, Shen X-B, Huang D-B, Jia W, Liu W-B, He Y-F. Peroxiredoxin 4

suppresses anoikis and augments growth and metastasis of hepatocellular carcinoma cells through the beta-catenin/ID2 pathway. Cell Oncol (Dordr). 2019;42(6):769-81.

16. Ummanni R, Mundt F, Pospisil H, Venz S, Scharf C, Barett C, et al. Identification of clinically relevant protein targets in prostate cancer with 2D-DIGE coupled mass spectrometry and systems biology network platform. Plos One. 2011;6(2):e16833.

17. Guo X, Noguchi H, Ishii N, Homma T, Hamada T, Hiraki T, et al. The Association of Peroxiredoxin 4 with the Initiation and Progression of Hepatocellular Carcinoma. Antioxid Redox Signal. 2019;30(10):1271-84.

18. Jianbo Zheng, Xin Guo, Akihiro Shioya, Takako Yoshioka, Kimikazu Matsumoto,Sohsuke Yamada, et al. Peroxiredoxin 4 promotes embryonal hepatoblastoma cell migration but induces fetal cell differentiation. Am J Transl Res. 2020; In Press.

19. Jia W, Chen P, Cheng Y. PRDX4 and Its Roles in Various Cancers. Technol Cancer Res Treat. 2019 Jan 1;18:1533033819864313.

20. Global Burden of Disease Cancer C, Fitzmaurice C, Allen C, Barber RM, Barregard L, Bhutta ZA, et al. Global, Regional, and National Cancer Incidence, Mortality, Years of Life Lost, Years Lived With Disability, and Disability-Adjusted Life-years for 32 Cancer Groups, 1990 to 2015: A Systematic Analysis for the Global Burden of Disease Study. JAMA Oncol. 2017;3(4):524-48.

21. Vincent RG, Pickren JW, Lane WW, Bross I, Takita H, Houten L, et al. The changing histopathology of lung cancer: a review of 1682 cases. Cancer. 1977;39(4):1647-55.

22. Pastor MD, Nogal A, Molina-Pinelo S, Carnero A, Paz-Ares L. Proteomic biomarkers in lung cancer. Clin Transl Oncol. 2013;15(9):671-82.

23. Hwang JA, Song JS, Yu DY, Kim HR, Park HJ, Park YS, et al. Peroxiredoxin 4 as an independent prognostic marker for survival in patients with early-stage lung squamous cell carcinoma. Int J Clin Exp Pathol. 2015;8(6):6627-35.

24. Lehtonen ST, Svensk A-M, Soini Y, Paakko P, Hirvikoski P, Kang SW, et al. Peroxiredoxins, a novel protein family in lung cancer. Int J Cancer. 2004;111(4):514-21.

25. Wei Q, Jiang H, Xiao Z, Baker A, Young MR, Veenstra TD, et al. Sulfiredoxin-Peroxiredoxin IV axis promotes human lung cancer progression through modulation of specific phosphokinase signaling. Proc Natl Acad Sci U S A. 2011;108(17):7004-9.

26. Jiang H, Wu L, Mishra M, Chawsheen HA, Wei Q. Expression of peroxiredoxin 1 and 4 promotes human lung cancer malignancy. Am J Cancer Res. 2014;4(5):445-60.

27. Shioya A, Guo X, Motono N, Mizuguchi S, Kurose N, Nakada S, et al. The Combination Of Weak Expression Of PRDX4 And Very High MIB-1 Labelling Index Independently Predicts Shorter Disease-free Survival In Stage I Lung Adenocarcinoma. Int J Med Sci. 2018;15(10):1025-34.

28. Mizutani K, Guo X, Shioya A, Zhang J, Zheng J, Kurose N, et al. The impact of PRDX4 and the EGFR mutation status on cellular proliferation in lung adenocarcinoma. Int J Med Sci. 2019;16(9):1199-206.

29. Shichita T, Hasegawa E, Kimura A, Morita R, Sakaguchi R, Takada I, et al.

Peroxiredoxin family proteins are key initiators of post-ischemic inflammation in the brain. Nat Med. 2012 Jun;18(6):911-7.

30. Ishii T, Warabi E, Yanagawa T. Novel roles of peroxiredoxins in inflammation, cancer and innate immunity. J Clin Biochem Nutr. 2012 Mar;50(2):91-105.

31. Yun HM, Park KR, Park MH, Kim DH, Jo MR, Kim JY, et al. PRDX6 promotes tumor development via the JAK2/STAT3 pathway in a urethane-induced lung tumor model. Free Radic Biol Med. 2015 Mar;80:136-44.

32. Stoner GD. Introduction to mouse lung tumorigenesis. Exp Lung Res. 1998 Jul-Aug;24(4):375-83.

33. Ding Y, Yamada S, Wang K-Y, Shimajiri S, Guo X, Tanimoto A, et al. Overexpression of peroxiredoxin 4 protects against high-dose streptozotocin-induced diabetes by suppressing oxidative stress and cytokines in transgenic mice. Antioxid Redox Signal. 2010;13(10):1477-90.

34. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature. 2008 Jul 24;454(7203):436-44.

35. Sica A, Allavena P, Mantovani A. Cancer related inflammation: the macrophage connection. Cancer Lett. 2008 Aug 28;267(2):204-15.

36. Pollard JW. Trophic macrophages in development and disease. Nat Rev Immunol. 2009 Apr;9(4):259-70.

37. Karin M, Lawrence T, Nizet V. Innate immunity gone awry: linking microbial infections to chronic inflammation and cancer. Cell. 2006 Feb 24;124(4):823-35.

38. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte

subsets: cancer as a paradigm. Nat Immunol. 2010 Oct;11(10):889-96.

39. Clear AJ, Lee AM, Calaminici M, Ramsay AG, Morris KJ, Hallam S, et al. Increased angiogenic sprouting in poor prognosis FL is associated with elevated numbers of CD163+ macrophages within the immediate sprouting microenvironment. Blood. 2010 Jun 17;115(24):5053-6.

40. Murdoch C, Muthana M, Coffelt SB, Lewis CE. The role of myeloid cells in the promotion of tumour angiogenesis. Nat Rev Cancer. 2008 Aug;8(8):618-31.

41. Lin EY, Li JF, Gnatovskiy L, Deng Y, Zhu L, Grzesik DA, et al. Macrophages regulate the angiogenic switch in a mouse model of breast cancer. Cancer Res. 2006 Dec 1;66(23):11238-46.

42. Lin EY, Nguyen AV, Russell RG, Pollard JW. Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. J Exp Med. 2001 Mar 19;193(6):727-40.

43. Zaynagetdinov R, Sherrill TP, Polosukhin VV, Han W, Ausborn JA, McLoed AG, et al. A critical role for macrophages in promotion of urethane-induced lung carcinogenesis. J Immunol. 2011 Dec 1;187(11):5703-11.

44. Fritz JM, Tennis MA, Orlicky DJ, Lin H, Ju C, Redente EF, et al. Depletion of tumor-associated macrophages slows the growth of chemically induced mouse lung adenocarcinomas. Front Immunol. 2014;5:587.

45. Apte RN, Dotan S, Elkabets M, White MR, Reich E, Carmi Y, et al. The involvement of IL-1 in tumorigenesis, tumor invasiveness, metastasis and tumor-host interactions. Cancer Metastasis Rev. 2006 Sep;25(3):387-408.

46. Narayan C, Kumar A. Constitutive over expression of IL-1beta, IL-6, NF-kappaB, and Stat3 is a potential cause of lung tumorgenesis in urethane (ethyl carbamate) induced Balb/c mice. J Carcinog. 2012;11:9.

47. Cuzzocrea S, Riley DP, Caputi AP, Salvemini D. Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury. Pharmacol Rev. 2001 Mar;53(1):135-59.

48. Hanaka T, Kido T, Noguchi S, Yamada S, Noguchi H, Guo X, et al. The overexpression of peroxiredoxin-4 affects the progression of idiopathic pulmonary fibrosis. BMC Pulm Med. 2019 Dec 30;19(1):265.

Figure legends

Figure 1. The expression of PRDX4 in Tg and non-Tg mice. A: No significant difference in the expression of PRDX4 was found in the initial lung specimens or lung tumors between Tg and non-Tg controls. After urethane stimulation, PRDX4 was obviously elevated in Tg mice in comparison to non-Tg controls. B: For Tg mice, serum hPRDX4 increased after urethane stimulation and decreased after tumor formation. No or very weak hPRDX4 was detected in the serum of non-Tg mice.

Figure 2. The overexpression of PRDX4 promotes lung tumor development. A: A gross view of a lung tumor. In comparison to non-Tg mice, increased numbers of tumors and larger tumors were found on the lung surface of Tg mice. B: A microscopic view of a lung tumor (hematoxylin and eosin staining). Tumor formation in Tg mice was increased in comparison to non-Tg mice. C: The effect of tumor development on mouse body weight. No significant difference was found between Tg mice and non-Tg controls during the urethane injection period. The body weight of Tg mice was significantly lower in comparison to non-Tg controls at the time of sacrifice (12 weeks after the last urethane injection). Data indicate the mean±SD. An independent-samples t-test was used for the analysis. *$p<0.05$.

Figure 3. The suppression of tumor apoptosis and enhancement of tumor proliferation in Tg mice. Immunohistochemical staining and Western blotting both showed that the expression of cleaved caspase-3 was significantly weaker in Tg tumors than in non-Tg controls. The expression of PCNA, analyzed by Western blotting, was stronger in Tg tumors than non-Tg tumors. Data are shown as the mean±SD. An

independent-samples t-test was used for the analysis.

Figure 4. Increased microvascular permeability and macrophage infiltration in Tg tumor. More microvessels permeated into the Tg tumors in comparison to non-Tg controls and more Mac-2 positive cells infiltrated the Tg tumors. There was no significant difference in CD3 T lymphocytes. Data are shown as the mean±SD. An independent-samples t-test was used for the analysis.

Figure 5. The elevated expression of MMP9 and IL-1β in Tg tumors. A: The expression levels of *MMP9* and *MMP13* mRNA in Tg tumors were significantly higher than in non-Tg tumor. The expression levels of *IL-1β*, *IL-6*, *TNF-α*, and *iNOS* in Tg tumors tended to be increased; however, the difference was not statistically significant. B: The expression levels of IL-1β and MMP9 were increased in Tg tumors. The phosphorylation of NF-κB p65 and c-jun in Tg tumors were enhanced in comparison to non-Tg controls. No significant difference was found in the expression of TNF-α or MMP3. Data are shown as the mean±SD. An independent-samples t-test was used for the analysis. $^{*}p<0.05$, $^{**}p<0.01$.

Figure 6. Inhibition of oxidative stress in Tg mice. A: The proportion of 8-OHdG-positive cells in Tg tumors was significantly lower than in non-Tg tumors. B: Serum malondialdehyde (MDA) in Tg mice was significantly lower than in non-Tg controls after tumor formation. Data are shown as the mean±SD. An independent-samples t-test was used for the analysis.