Peroxiredoxins, thioredoxin, and Y-box-binding protein-1 are involved in the pathogenesis and progression of dialysis-associated renal cell carcinoma

伏見, 文良

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Corresponding Author:	Yoshinao Oda, Ph.D., M.D. JAPAN
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	
Corresponding Author's Secondary Institution:	
First Author:	Fumiyoshi Fushimi, M.D., Ph.D.
First Author Secondary Information:	
Order of Authors:	Fumiyoshi Fushimi, M.D., Ph.D.
	Kenichi Taguchi, M.D., Ph.D.
	Hiroto Izumi, M.D., Ph.D.
	Kimitoshi Kohno, M.D., Ph.D.
	Michihiko Kuwano, M.D., Ph.D.
	Mayumi Ono, Ph.D
	Yutaka Nakashima, M.D., Ph.D.
	Tetsuro Takesue, M.D.
	Seiji Naito, M.D., Ph.D.
	Yoshinao Oda, Ph.D., M.D.
Order of Authors Secondary Information:	
Abstract:	Patients with end-stage renal disease are exposed to increased oxidative stress and impairment of antioxidant mechanisms. We focused on dialysis renal cell carcinoma (RCC), including epithelial hyperplasia in acquired cystic disease of the kidney (ACDK). We attempted to obtain insight into the carcinogenesis and tumor progression in terms of cellular defense mechanisms associated with oxidative stress, by investigating the expression of antioxidant proteins by immunohistochemistry. We evaluated retrospectively 43 cases of dialysis RCC and, as a control group, 49 cases of sporadic RCC. Peroxiredoxin (Prx) 1,3,4,5,6 expression in dialysis RCC was positively correlated with the duration of dialysis. In epithelial hyperplasia in 17 cases of acquired cystic disease of the kidney, Prxs and thioredoxin were highly expressed. Moreover, in dialysis RCC Prx 3,4,5 immunoreactivity and nuclear expression of Y-box-binding protein-1 were higher than in sporadic RCC. In dialysis RCC, Prx 3,4,5 immunoreactivity positively correlated with the Fuhrman nuclear grade. These data suggest that oxidative stress during dialysis enhances antioxidant activity with an inhibiting effect on carcinogenesis. Once a cancer has developed, antioxidant activity might have a stimulating effect on progression of dialysis RCC.
Response to Reviewers:	Fred T. Bosman、MD PhD EDITOR-in-Chief Virchows Archiv

May 29 2013

Dear Dr. Bosmant

Thank you very much for your letter and the reviewers' comments on our manuscript entitled "Peroxiredoxins, thioredoxin and Y-box-binding protein-1 expression in renal cell carcinoma arising in patients on dialysis" by Fumiyoshi Fushimi et al. (manuscript number: VIAR-D-13-00106). The comments offered by the reviewers were very helpful and we have revised our manuscript to incorporate the reviewers' suggestions as follows:

Comments and our replies
Reviewer comments:
Reviewer #1 :
1. For the experiments the authors have chosen immunohistochemistry, which seems to work well on their specimen. Nevertheless it is discutable to address complex mechanisms like oxidative stress via immunohistochemistry only. As a result, the data is partially convincing, methods other than immunohistochemistry should have been used.

Reply:
As you indicated, it would be ideal to analyze the expression of antioxidant proteins by different techniques, such as western blotting and RT-PCR, in addition to the immunohistochemistry. Frozen sections are unavailable, however, so we have no choice but to choose immunohistochemistry only. In addition, immunohistochemistry was evaluated thoroughly by two pathologists to insure accuracy of interpretation.

We added the following sentence.
[Material and methods]
Page 7 line 29
Two pathologists (FF, KT) evaluated the immunohistochemistry to insure accuracy of interpretation.

2. I do not think that the Fuhrman grade is the best indicator for prognosis, especially as these groups also contained other renal cell carcinomas than clear cell neoplasms.

Reply:
It is generally agreed that nuclear grading, such as Fuhrman grade, is important in predicting prognosis in patients with renal cell carcinoma of clear cell neoplasms. However, as you noted, Fuhrman grade is not a global prognostic indicator in whole renal cell carcinomas.

We corrected the manuscript according to your suggestion:
[Result]
Page10 line 24
which is one of the major prognostic features of RCC
Page13 line 47
The Fuhrman nuclear grade is one of the important prognostic features of RCC
Page15 line 55
which is one of the major prognostic features of RCC

3. It would have been interesting to make a difference between Type 1 and 2 papillary carcinomas, and say a word about the 5 other cases

Reply:
We classified RCCs according to the 1997 UICC/ AJCC consensus and WHO (2004) classification systems. In dialysis RCC, however, clear-cut classification is difficult in many cases. In particular, type 1 and type 2 papillary RCC were not readily distinguished in histology. The tumors that morphologically did not fit the criteria for AJCC consensus and WHO (2004) classification systems were designated mixed type papillary RCC in reference to Yang's paper (Cancer Res.2005;65:5628-37).

We revised Table 2 in histology, according to your suggestion and in reference to the 1997 UICC/ AJCC consensus and WHO (2004) classification systems.

We added the following sentence.
[Material and methods]
Page 6 line 26
RCCs were classified according to the 1997 UICC/ AJCC consensus and WHO (2004) classification systems [16].

Page 8 line 26
In dialysis RCC, however, clear-cut classification is difficult in many cases. In particular, type 1 and type 2 papillary RCC were not readily distinguished in histology. The tumors that morphologically did not fit the criteria for AJCC consensus and WHO (2004) classification systems were designated mixed type papillary RCC in reference to Yang's paper [21]. There was no definite type 1 papillary RCC in dialysis RCC.

[Results]
Page 10 line 20
In dialysis RCC, there was no definite difference between type 2 and mixed type papillary RCC in terms of Prxs, TRX and YB-1 immunoreactivity.

Reviewer #2:
The paper may be improved with some corrections.
.
1. Last sentence of abstract and discussion (conclusion) needs to be reworded to make it more understandable

Reply:
We corrected the last sentence of the abstract and discussion as follows.
[Abstract]
Page 3 line 44
These data suggest that oxidative stress on dialysis could enhance antioxidant activity to prevent carcinogenesis. After carcinogenesis, antioxidant activity might create an advantageous phenotype to promote and progress dialysis RCC.

[Discussion]
Page 15 line 58
The findings of this study suggest that oxidative stress on dialysis could enhance antioxidant activity to prevent carcinogenesis. After carcinogenesis, antioxidant activity might create an advantageous phenotype to promote and progress dialysis RCC.

2. Material and methods, and results(also in Table 1):
-The authors include RCC controls and RCC from ACDK. There is just a basic comments on types. There were any of these recently described entities of RCC reported in ACDK patients? Did they have any difference in IHC expression of the markers? In papillary RCC, there were type 1 or Type 2 or mixed forms?. There were any unclassified RCC?. Pathologic description of sample cases should be improved in both sample and control group.

Reply-1: (Recently described entities of RCC reported in ACDK patients)
We revised Table 2 in histology, according to your suggestion and in reference to Tickoo's paper (Am J Surg Pathol 2006;30:141-153).

We added the following sentences on discussion
[Discussion]
Page 15 line 38
It is reported that RCC arising in a background of ACDK shows morphologic features that are not seen in sporadic RCC. We have also classified histological subtypes of dialysis related RCC based on Tickoo's paper [31] (Table 2). Two types of RCC were newly designated; acquired cystic disease-associated RCC (ACD-associated RCC) in five cases and clear cell papillary RCC in one case. ACD-associated RCC is characterized by eosinophilic cytoplasm with Fuhrman's grade 3 nuclei, frequent

association with intratumoral oxalate crystals, and various combinations of acinar, solid alveolar, solid sheet-like, micro-cystic, macro-cystic, and papillary architecture. Prominent papillary architecture in our cases led us to classify them as papillary RCC. Various combinations of architecture led us to interpret them as RCC, unclassified. All Prxs and TRX immunoreactivity in 5 cases of ACD-associated RCC were high and the mean nuclear expression of YB-1 was 30%. ACD-associated RCC showed immune profiles similar to epithelial hyperplasia in ACDK. This may imply that epithelial hyperplasia in ACDK is putative precursor lesion of ACD-associated RCC.

Reply-2: (Pathologic description of sample cases, including papillary RCC)
We classified RCCs according to the 1997 UICC/ AJCC consensus and WHO (2004) classification systems. Papillary RCCs were classified, according to your suggestion and in reference to Yang's paper (Cancer Res.2005;65:5628-37).
Please refer to our reply to Reviewer # 1

3. There were differences in IHC expression of markers in non-neoplastic kidney tissues in both groups?

Reply:
We think there were no differences in IHC expression of markers in non-neoplastic kidney tissue in both groups, although precise comparison is difficult in some cases, especially in advanced cases of ACDK.
IHC expression of markers in non-neoplastic kidney tissues in both groups was given in page 8 line 41
Additionally, IHC expression of markers in non-neoplastic kidney tissues in ACD was given in page 11 line 1.

We corrected the manuscript to make it clear that there is no definite difference in IHC expression of markers in non-neoplastic kidney tissues between the two groups.
[Result]
Page 8 line 41
In non-neoplastic renal tissue in both sporadic and dialysis RCC, all Prxs and TRX showed varying immunoreactivity in the cytoplasm of proximal or distal renal tubules.

4. Pathologic characteristics of hyperplastic lesions could be better described to assist the reader in getting familiar with them.

Reply
We changed the sentence as follows according to your suggestion:
[Introduction]
Page 4 line 49
Cysts in ACDK are lined by flattened to cuboidal epithelium, and in most cases, focal epithelial hyperplastic proliferation can be seen. This epithelial hyperplasia arising in ACDK may pose an increased risk for the development of RCC [6].

5. Fig 1b shows nuleolar prominence. Were sample cases evaluated by gender, i.e. Could it be cases of familiar genetic alteration in this group. Any association with leiomyomata

Reply
We selected the sporadic and dialysis related cases, as far as we can tell, which have no family history of renal tumor. We believe therefore that there is no hereditary leiomyomatosis in our cases including the case in Fig. 1b.

We added a sentence to make it clear that we have excluded hereditary RCC.
[Material and methods]
Page 6 line26
There was no family history of renal neoplasm in all 92 cases.

Finally, we added three new references and quotation numbers were altered

	accordingly. [16] Eble JN, Sauter G, Epstein JI, Sesterhenn IA eds.(2004) World Health Organization Classification of Tumors. Pathology and genetics of tumors of the Urinary System and Male Genital Organs. IARC Press: Lyon. [21] Yang XJ, Tan MH, Kim HL et al (2005) A molecular classification of papillary renal cell carcinoma. 65:5628-37. [31] Tickoo SK, dePeralta-Venturina MN, Harik LR et al (2006) Spectrum of epithelial neoplasm in end stage renal disease; an experience from 66 tumor bearing kidneys with emphasis on histologic patterns distinct from those in sporadic adult renal neoplasia. Am J Surg Pathol. 30:141-153. We thank you for your very helpful suggestions which have improved our report. Your kind consideration of this revision for publication in Virchows Arch would be appreciated. Sincerely yours, Yoshinao Oda Fumiyoshi Fushimi

Peroxiredoxins, thioredoxin and Y-box-binding protein-1 are involved in the pathogenesis and progression of dialysis associated renal cell carcinoma

Fumiyoshi Fushimi,[1] Kenichi Taguchi,[2] Hiroto Izumi,[3] Kimitoshi Kohno,[3] Michihiko Kuwano,[4] Mayumi Ono,[4] Yutaka Nakashima,[5] Tetsuro Takesue,[5] Seiji Naito,[6] and Yoshinao Oda,[1]

[1]Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Higashi-ku, Fukuoka, Japan

[2]Department of Pathology, National Kyushu Cancer Center, Minami-ku, Fukuoka, Japan

[3]Department of Molecular Biology, University of Occupational and Environmental Health, Kitakyushu, Fukuoka, Japan

[4]Department of Pharmaceutical Oncology and Laboratory of Molecular Cancer Biology, Graduate School of Pharmaceutical Sciences, Kyushu University, Higashi-ku, Fukuoka, Japan

[5]Department of Pathology and Department of Urology, Fukuoka Red Cross Hospital, Minami-ku, Fukuoka, Japan

[6]Department of Urology, Graduate School of Medical Sciences, Kyushu University, Higashi-ku, Fukuoka, Japan

Corresponding author and requests for reprints: Yoshinao Oda, Department of Anatomic Pathology, Pathological Sciences, Graduate School of Medical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan

E-mail: oda@surgpath.med.kyushu-u.ac.jp

Tel: +81-92-642-6061, FAX: +81-92-642-5968

Abstract

Patients with end-stage renal disease are exposed to increased oxidative stress and impairment of antioxidant mechanisms. We focused on dialysis renal cell carcinoma (RCC), including epithelial hyperplasia in acquired cystic disease of the kidney (ACDK). We attempted to obtain insight into the carcinogenesis and tumor progression in terms of cellular defense mechanisms associated with oxidative stress, by investigating the expression of antioxidant proteins by immunohistochemistry. We evaluated retrospectively 43 cases of dialysis RCC and, as a control group, 49 cases of sporadic RCC. Peroxiredoxin (Prx) 1,3,4,5,6 expression in dialysis RCC was positively correlated with the duration of dialysis. In epithelial hyperplasia in 17 cases of acquired cystic disease of the kidney, Prxs and thioredoxin were highly expressed. Moreover, in dialysis RCC Prx 3,4,5 immunoreactivity and nuclear expression of Y-box-binding protein-1 were higher than in sporadic RCC. In dialysis RCC, Prx 3,4,5 immunoreactivity positively correlated with the Fuhrman nuclear grade. These data suggest that oxidative stress during dialysis enhances antioxidant activity with an inhibiting effect on carcinogenesis. Once a cancer has developed, antioxidant activity might have a stimulating effect on progression of dialysis RCC.

Key words: oxidative stress, peroxiredoxin, Y-box-binding protein-1, renal cell carcinoma, acquired cystic disease of kidney

Introduction

The association between dialysis patients and both increased oxidative stress and the impairment of the antioxidant defense mechanism is well-established [1]. The increased tendency of dialysis patients to develop renal cell carcinoma (RCC) compared to the general population is reported also, although its carcinogenesis remains poorly understood [2]. In a previous investigation, we evaluated oxidative stress by immunohistochemical expression of 8-oxoguanine and found a higher accumulation of 8-oxoguanine in dialysis RCC than in sporadic RCC [3]. This result suggests in dialysis RCC the level of oxidative stress may be correlated with carcinogenesis.

DNA oxidation may be an important factor in carcinogenesis. Oxidative stress is known to modulate cell proliferation and apoptosis, and induce synthesis of the growth factors that play an important role in tumor promotion and progression [4]. Antioxidant enzymes regulate the cellular redox state and constitute the major cellular protection against oxidative stress. Oxidant-antioxidant balance, therefore, may be important not only for carcinogenesis but also for tumor promotion and progression. Moreover, in most instances, dialysis RCC is considered to develop in association with acquired cystic disease of the kidney (ACDK) [5]. Cysts in ACDK are lined by flattened to cuboidal epithelium, and in most cases, focal epithelial hyperplastic proliferation can be seen. This epithelial hyperplasia arising in ACDK may pose an increased risk for the development of RCC [6]. In hyperplasia associated with dialysis and/or oxidative stress,

there may be a link between DNA damage and carcinogenesis.

Peroxiredoxin isoforms (Prxs) and thioredoxin (TRX) are ubiquitously distributed in all organisms including bacteria, plants and animals [7, 8]. Prxs and TRX are antioxidant enzymes that act as peroxidases, and have emerged as key molecules in the antioxidant defense mechanism. In recent years Prxs and TRX have been shown to have roles in cancer prevention, but they have diverse functions and reports link them both to the prevention and the promotion of the cancer and their exact role is still controversial [9-13].

Y-box-binding protein-1 (YB-1) is a transcription factor that plays important roles in cell proliferation, DNA replication and drug resistance. Cytoplasmic YB-1 is reported to translocate to the nucleus during UVA-induced oxidative stress [14]. YB-1 is also expected to act on recognition of oxidative DNA damage in the DNA repair pathway [15].

In this report we focused on dialysis RCC, including epithelial hyperplasia arising in ACDK. We attempted to obtain some insights into carcinogenesis and tumor progression in term of the cell's defense mechanism associated with oxidative stress by investigating immunohistochemical Prxs, TRX and YB-1 expression.

Materials and methods

Patients

A total of 43 cases of renal neoplasm in patients on dialysis between 1992 and 2008 were obtained from the Department of Anatomic Pathology, Kyushu University and from the Department of Pathology, Fukuoka Red Cross Hospital. As a control group, 49 cases of sporadic RCC were selected. Because of specimen deterioration, the number of cases decreased to 38 cases of dialysis RCC and 49 cases of sporadic RCC. Five cases of dialysis RCC were newly obtained from the Department of Anatomic Pathology of Kyushu University between 2006 and 2008. The five new cases were diagnosed as described in our previous report [3]. RCCs were classified according to the 1997 UICC/ AJCC consensus and WHO (2004) classification systems [16]. There was no family history of renal neoplasm in all 92 cases. The institutional review board at Kyushu University approved this study (permission code: 24-59).

Immunohistochemistry

Immunohistochemical staining was performed using 10% formalin-fixed, paraffin-embedded tissue sections using the avidin–biotin–peroxidase method, with positive and negative controls. Details of the primary antibodies are summarized in Table 1. Anti-Prx5 [17] and anti-YB-1 [18] antibodies were prepared as previously described.

Interpretation and scoring of immunohistochemical preparations

Immunoreactivity was interpreted as positive based on the presence of cytoplasmic staining for Peroxiredoxin isoforms (Prxs) and Thioredoxin (TRX). A proportion score was assigned to represent the estimated proportion of positively stained tumor cells (0 = none, 1 = <1/100, 2 = 1/100 to 1/10, 3 = 1/10 to 1/3, 4 = 1/3-2/3, 5 = >2/3). The average estimated intensity of staining in positive cells was given as an intensity score (0 = none, 1 = weak, 2 = intermediate, 3 = strong). The proportion score and the intensity score were added to get a total score. The immunohistochemical expression status was trichotomized based on the total score, according to our previously report: negative (-), 0; low positive (low +), 2 to 4; and high positive (high +); 5 to 8 [19]. Nuclear immunohistochemical expression of YB-1 protein was evaluated as a percentage of positively stained nuclei in a selected hot spot [20]. Two pathologists (FF, KT) evaluated the immunohistochemistry to insure accuracy of interpretation.

Statistical analysis

The clinicopathological factors were analyzed using the chi-square test or unpaired student’s t-test. The Yates Chi-square test was used to assess the significance of the differences in the immunoreactivity between the expressions of Prxs and TRX in dialysis RCC, sporadic RCC and hyperplastic epithelium in ACDK. The unpaired t-test

was used to assess the significance of the differences between the nuclear immunohistochemical expression of YB-1 in dialysis RCC, sporadic RCC and hyperplastic epithelium in ACDK. The Spearman rank method was used to calculate correlation coefficients between duration of dialysis and immunohistochemical results in dialysis RCC. P values less than .05 were considered statistically significant.

Results

Clinicopathological findings

The clinicopathological data on 43 cases of dialysis RCC and 49 cases of sporadic RCC are summarized in Table 2. The 92 patients with renal neoplasm analyzed in this study had an age range of 37-83 years. Histologically, the frequency of papillary type was higher in dialysis RCC (10/43 cases: 23.3%) than in sporadic RCC (5/49 cases: 10.2%), but was not statistically significant (p=0.159). In dialysis RCC, however, clear-cut classification is difficult in many cases. In particular, type 1 and type 2 papillary RCC were not readily distinguished in histology. The tumors that morphologically did not fit the criteria for AJCC consensus and WHO (2004) classification systems were designated mixed type papillary RCC in reference to Yang's paper [21]. There was no definite type 1 papillary RCC in dialysis RCC. The other clinicopathological findings are almost the same as described in our previous report [3].

Immunohistochemical expression of Prxs, TRX and YB-1 in sporadic and dialysis RCC

In non-neoplastic renal tissue in both sporadic and dialysis RCC, all Prxs and TRX showed varying immunoreactivity in the cytoplasm of proximal or distal renal tubules. Cells of collecting ducts and glomerular epithelial cells showed no immunoreactivity whatsoever. Prxs and TRX immunoreactivity in RCC were shown in the tumor cytoplasm, regardless of isoforms (Figure 1a, 1b). The results of immunohistochemical study for Prxs, TRX and YB-1 are summarized in Table 3. All Prxs and TRX immunoreactivity in dialysis RCC have higher scores than in sporadic RCC. In particular, expressions of Prx3($p=0.037$), Prx4($p=0.046$), Prx5($p=0.021$) were significantly higher in dialysis RCC (Table 4).

YB-1 protein expression was observed in both cytoplasm and nucleus or only in cytoplasm in renal cell carcinoma (Figure 2). Dialysis RCC showed statistically higher nuclear immunohistochemical expression of YB-1(39.2%, mean), compared to the sporadic RCC (12.8%, mean) (Table 4, $p<0.001$).

The relationship between the duration of dialysis and immunohistochemical expression in dialysis RCC

The mean duration of dialysis was 146 months in patients with dialysis RCC who had an accurate dialysis record (n=34). The mean duration of dialysis was 16

months in patients with no cysts (n=6), 174 months in those with acquired cystic disease (n=28) and was statistically different ($p<0.001$). Immunohistochemical results with reference to the duration of dialysis for over 120 months and under 120 months are summarized in Table 5. The mean total scores of all Prxs were higher in patients with a long duration of dialysis (>120 months). Moreover, positive correlations between the duration of dialysis and Prx 1,3,4,5,6 expression were observed in dialysis RCC, as evaluated by Spearman's rank method. In contrast, Prx2 expression, TRX expression and nuclear expression of YB-1 were not correlated with duration of dialysis.

Prxs TRX and YB-1 immunoreactivity and its correlation with clinicopathological factors

Table 6 shows the correlations between Prxs, TRX and YB-1 immunoreactivity, and clinicopathological data in sporadic and dialysis RCC. The clear cell type RCC and papillary type RCC were compared. The papillary type showed higher immunoreactivity of Prxs compared to the clear cell type in both dialysis RCC and sporadic RCC ($p<0.001$). In dialysis RCC, there was no definite difference between type 2 and mixed type papillary RCC in terms of Prxs, TRX and YB-1 immunoreactivity. In dialysis RCC, the immunoreactivity of all the six Prxs was positively correlated with the Fuhrman nuclear grade, which is one of the major prognostic features of RCC. On the other hand, in sporadic RCC, no correlation was

observed between Prxs immunoreactivity and the Fuhrman nuclear grade. The other clinicopathological factors such as stage and tumor diameter had no correlation with Prxs immunoreactivity. No significant correlation was observed between nuclear expression of YB-1 and clinicopathological factors.

Epithelial hyperplasia in acquired cystic disease of kidney

In 43 Dialysis RCC, at least 29 were accompanied by acquired cystic disease of the kidney (ACDK). Epithelial hyperplasia was detected in 17/29 cases of ACDK (Figure 3). Epithelium that lines the ACDK is mostly flattened, and hyperplastic epithelium is irregularly distributed. The results of immunohistochemical study for Prxs, TRX and YB-1 in epithelial hyperplasia in ACDK are summarized in Table 3. In all 29 cases of ACDK, the lining flat epithelial cells show variable immunoreactivity of Prxs and TRX from negative to high positive.

On the other hand, in epithelial hyperplasia in 17 cases of ACDK, Prxs and TRX immunoreactivity was high except in one case in which Prx1,5,6 and TRX expression was not observed. All Prxs and TRX immunoreactivity in epithelial hyperplasia of ACDK had significantly higher scores compared with those in both sporadic RCC and dialysis RCC (Table 3, Table 4). The mean nuclear expression of YB-1 in epithelial hyperplasia in ACDK was 39.0%, compared with 12.8% in sporadic RCC and was statistically different ($p<0.001$).

Discussion

Several characteristic genetic alterations have been found to be associated with the carcinogenesis of renal cell carcinoma (RCC), such as mutations of the VHL gene. To date, however, there is little evidence of specific genetic mutation in patients on dialysis, although a few reports show mutations of the VHL gene [22]. It is reported that the genetics of renal cell carcinoma associated with acquired cystic renal disease may differ from those occurring in the general population [2]. The histological types of dialysis RCC is also distinct from that of sporadic RCC, especially in patients with a duration of dialysis for more than 120 months [23]. Clear cell type RCC was the predominant histological type in patients with a duration of dialysis of less than 120 months, while the predominance of clear cell RCC decreased in those on dialysis for more than 120 months. The risk of RCC is considered to be higher in patients with a longer duration of dialysis. Several pathological mechanisms could contribute to this higher risk, including the increased synthesis of reactive oxygen species. Dialysis induces oxidative stress and may be associated with oxidative DNA damage and carcinogenesis. We hypothesized that oxidative stress induces carcinogenesis in dialysis patients, and found significantly higher levels of 8-oxoguanine in dialysis RCC than in sporadic RCC [3]. The process of carcinogenesis by oxidative stress is accompanied by

cumulative mutations in genetic pathways that are advantageous to carcinogenesis. The carcinogenesis process by oxidative stress, therefore, is thought to involve many genes and is a result of multistage mutagenesis. Oxidative stresses have a complex nature and oxidant-antioxidant balance might be important for carcinogenesis and tumor progression. The expression of antioxidant enzyme or stress protein in response to oxidative stress is to be expected.

Prxs and TRX are antioxidant enzymes and they also modulate intracellular signaling pathways related to apoptosis and cell proliferation. YB-1 is reported to be a transcriptional regulatory factor for several genes including the multidrug resistance 1 gene in human malignancy [24]. Although, to the best of our knowledge, there are no reports that YB-1 directly regulates antioxidant enzymes, such as Prxs and TRX, the association between YB-1 and oxidative stress has been reported [14, 15]. In the present study, Prx 1,3,4,5,6 expression of dialysis RCC was positively related with the duration of dialysis. This result suggests that the longer duration of dialysis causes the higher oxidative stress and antioxidative activities. ACDK bears a strong relationship to the duration of dialysis, and it is reported as being present in more than 90% of patients at 120 months or more of dialysis [25]. Epithelial hyperplasia arising in ACDK is a characteristic feature of kidneys on dialysis and has been implicated in the pathogenesis of renal cell carcinoma [6]. Our results showed Prxs and TRX immunoreactivity and nuclear expression of YB-1 in epithelial hyperplasia in ACDK were at high levels.

Moreover, Prx 3,4,5 immunoreactivity and nuclear expression of YB-1 in dialysis RCC were at higher levels compared with those in sporadic RCC. Therefore, expression of Prx 3,4,5 and YB-1 in epithelial hyperplasia in ACDK and dialysis RCC may be a response to the stimuli with oxidative stress. In dialysis RCC, all the six Prxs' immunoreactivity were positively correlated with the Fuhrman nuclear grade, which is defined as four nuclear grades in the order of increasing nuclear size, irregularity and nucleolar prominence [26]. The Fuhrman nuclear grade is one of the important prognostic features of RCC [27]. Our data therefore suggest that Prxs, TRX and YB-1 may have effects of the promotion and progression of dialysis RCC. In Japan, patients with dialysis have been closely monitored for dialysis RCC. Early detection is expected from the fact that in most cases the AJCC stage is T1 and the diameter of most dialysis RCC tumors is less than 4cm. It is speculated that AJCC stage and tumor diameter are not useful prognostic features and the Fuhrman nuclear grade may be the most useful prognostic feature in the case of dialysis RCC in Japan.

Our results also suggest the bipolar roles of antioxidant defense mechanisms in carcinogenesis and tumor progression (Figure 4). Prxs, TRX and YB-1 in dialysis RCC may not only be an indicator of oxidative stress, but may also represent an adaptive response by tumor cells to adjust and survive the oxidative environment. Since Prxs, TRX and YB-1 are highly expressed in epithelial hyperplasia in ACDK, it is expected that antioxidants are associated with anti-cancerous effects in the early

development of the carcinogenesis (Figure 4a). But once carcinogenesis occurs, constant oxidative stress may work to aid tumor protection in maintaining the expression of antioxidant proteins, such as Prxs, TRX and YB-1, compared to the general population (Figure 4b). Prxs are reported to play a role in cell proliferation, differentiation, immune response, protection of oxidant-sensitive proteins, regulation of cellular hydrogen peroxide and control of apoptosis, processes involving in oxidative stress-related cellular signaling [28]. Our results suggest that constant oxidative stress, such as dialysis, could enhance antioxidant activity to prevent carcinogenesis, but after carcinogenesis, antioxidant activity leads to the acquisition of advantageous phenotype which promotes and progresses the cancer. The functions of Prxs include not only the detoxification of peroxide, but also the increase of cell survival and proliferation. Protection against apoptotic stimuli is one possible explanation of the increased cell survival and proliferation. Our results show that Prx 3,4,5 especially may play a very specific role in the carcinogenesis and tumor progression. It is reported that the expression of peroxiredoxins, especially Prx 3,4,5 are increased in breast malignancy, in accordance with our results [29]. However, the significance of specific expression of Prx isoforms remains unresolved and further research is needed. It is reported that Prxs could be induced by irradiation, which also induce oxidative stress [30]. Its expression can be taken as an adaptive response and could be associated with the resistance of tumor cells to oxidant-generating irradiation. The nuclear expression of YB-1 is

reported to be associated with poor prognosis in cancer patients. Since YB-1 is a regulatory factor for multidrug resistance 1 gene, it is considered likely that YB-1 is responsible for treatment failure in cancer patients. Our study joins others in highlighting the complex nature of adaptive response in relation to carcinogenesis.

It is reported that RCC arising in a background of ACDK shows morphologic features that are not seen in sporadic RCC. We have also classified histological subtypes of dialysis related RCC based on Tickoo's paper [31] (Table 2). Two types of RCC were newly designated; acquired cystic disease-associated RCC (ACD-associated RCC) in five cases and clear cell papillary RCC in one case. ACD-associated RCC is characterized by eosinophilic cytoplasm with Fuhrman's grade 3 nuclei, frequent association with intratumoral oxalate crystals, and various combinations of acinar, solid alveolar, solid sheet-like, micro-cystic, macro-cystic, and papillary architecture. Prominent papillary architecture in our cases led us to classify them as papillary RCC. Various combinations of architecture led us to interpret them as RCC, unclassified. All Prxs and TRX immunoreactivity in 5 cases of ACD-associated RCC were high and the mean nuclear expression of YB-1 was 30%. ACD-associated RCC showed immune profiles similar to epithelial hyperplasia in ACDK. This may imply that epithelial hyperplasia in ACDK is putative precursor lesion of ACD-associated RCC.

In conclusion, we analyzed dialysis RCC, including epithelial hyperplasia arising in ACDK, by investigating immunohistochemical Prxs, TRX and YB-1

expression. All Prxs, TRX and YB-1 expression in dialysis RCC, including epithelial hyperplasia arising in ACDK, are at higher levels compared with that in sporadic RCC. In dialysis RCC, Prx 3,4,5 immunoreactivity was positively correlated with the Fuhrman nuclear grade, which is one of the major prognostic features of RCC. The findings of this study suggest that oxidative stress on dialysis could enhance antioxidant activity to prevent carcinogenesis. After carcinogenesis, antioxidant activity might create an advantageous phenotype to promote and progress dialysis RCC.

Acknowledgements

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Disclosure/conflict of interest

None.

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Figure legends

Figure 1

Immunohistochemical expression of Peroxiredoxin 3 and Thioredoxin in dialysis RCC.

(a) Clear cell RCC of a 59-year-old man shows strong cytoplasmic immunoreactivity for Peroxiredoxin 3 (score 8). (b) Papillary RCC of a 68-year-old female shows strong cytoplasmic immunoreactivity for Thioredoxin (score 7).

Figure 2

(a) Nuclear staining and (b) cytoplasmic staining of YB-1 protein**.**

Figure 3

Epithelial hyperplasia in acquired cystic disease of kidney and immunohistochemical expression of Peroxiredoxin 3 and Peroxiredoxin 5

(a) The cysts in acquired cystic disease of the kidney of a 70-year-old female are lined by flattened epithelium in the right and hyperplastic papillary epithelium in the left.

(b) Hyperplastic epithelium in acquired cystic disease of the kidney of a 53-year-old female shows strong cytoplasmic immunoreactivity for Peroxiredoxin 3 (score 8).

(c) Hyperplastic epithelium in acquired cystic disease of the kidney of a 44-year-old male shows strong cytoplasmic immunoreactivity for Peroxiredoxin 5 (score 8).

Figure 4

Potential mechanisms of antioxidant activity in the development of dialysis RCC

(a) Dialysis induces oxidative stress. Constant oxidative stress can result in cumulative DNA damage, which may be prevented by antioxidant proteins, such as Prxs.

(b) Constant oxidative stress induces the expression of antioxidant proteins, which can support the survival and growth of cancer cells.

Figure 1a
Click here to download high resolution image

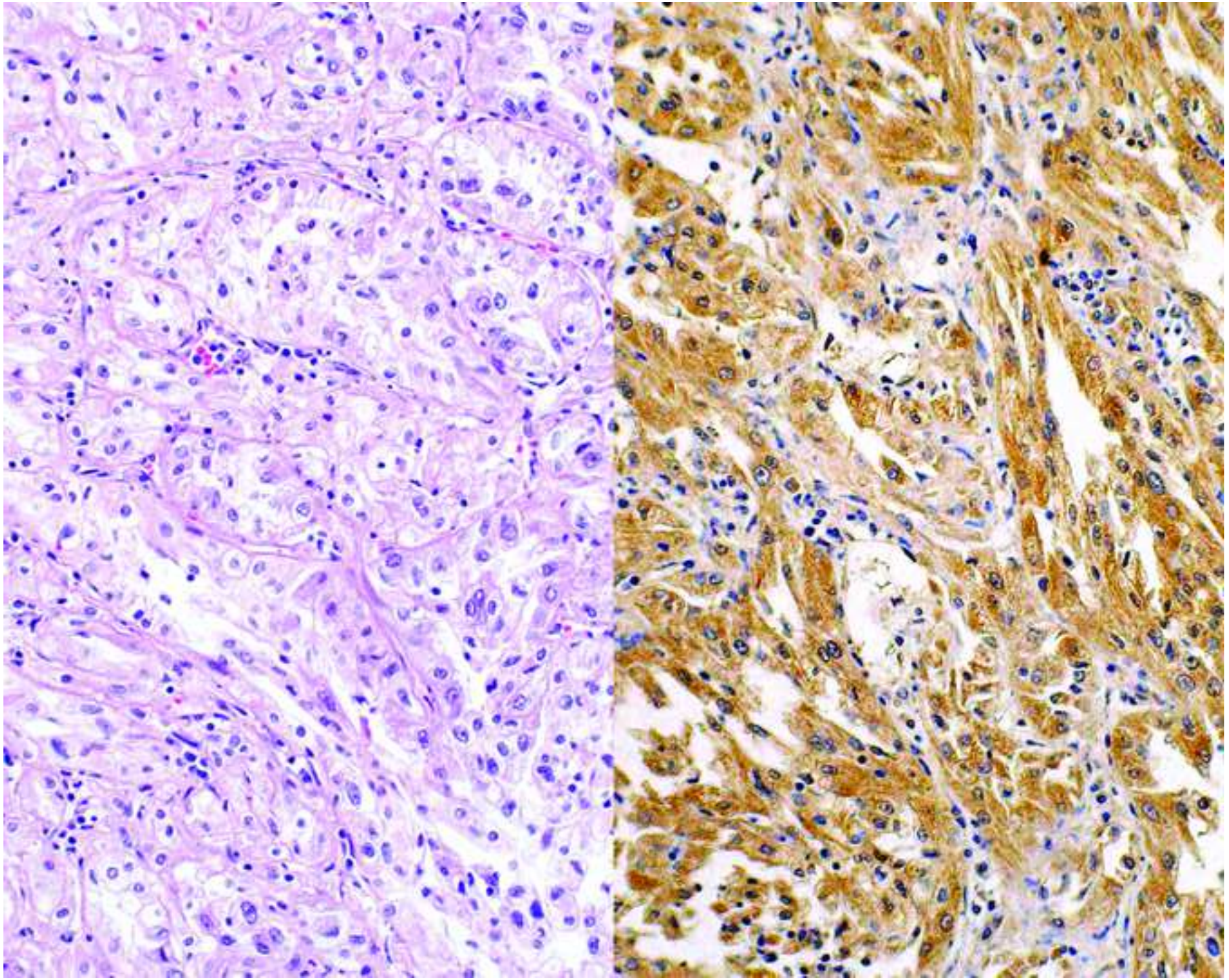

Figure 1b

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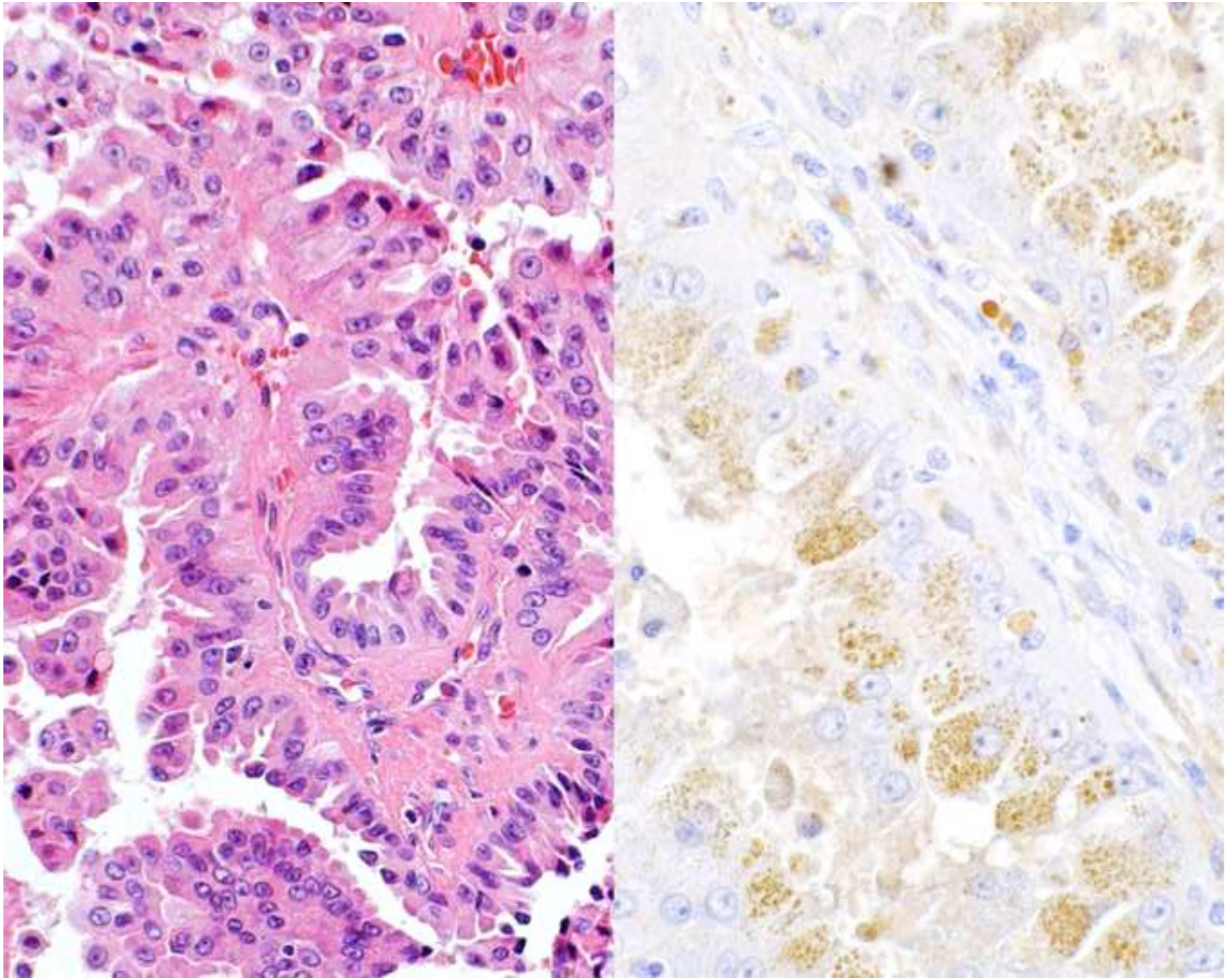

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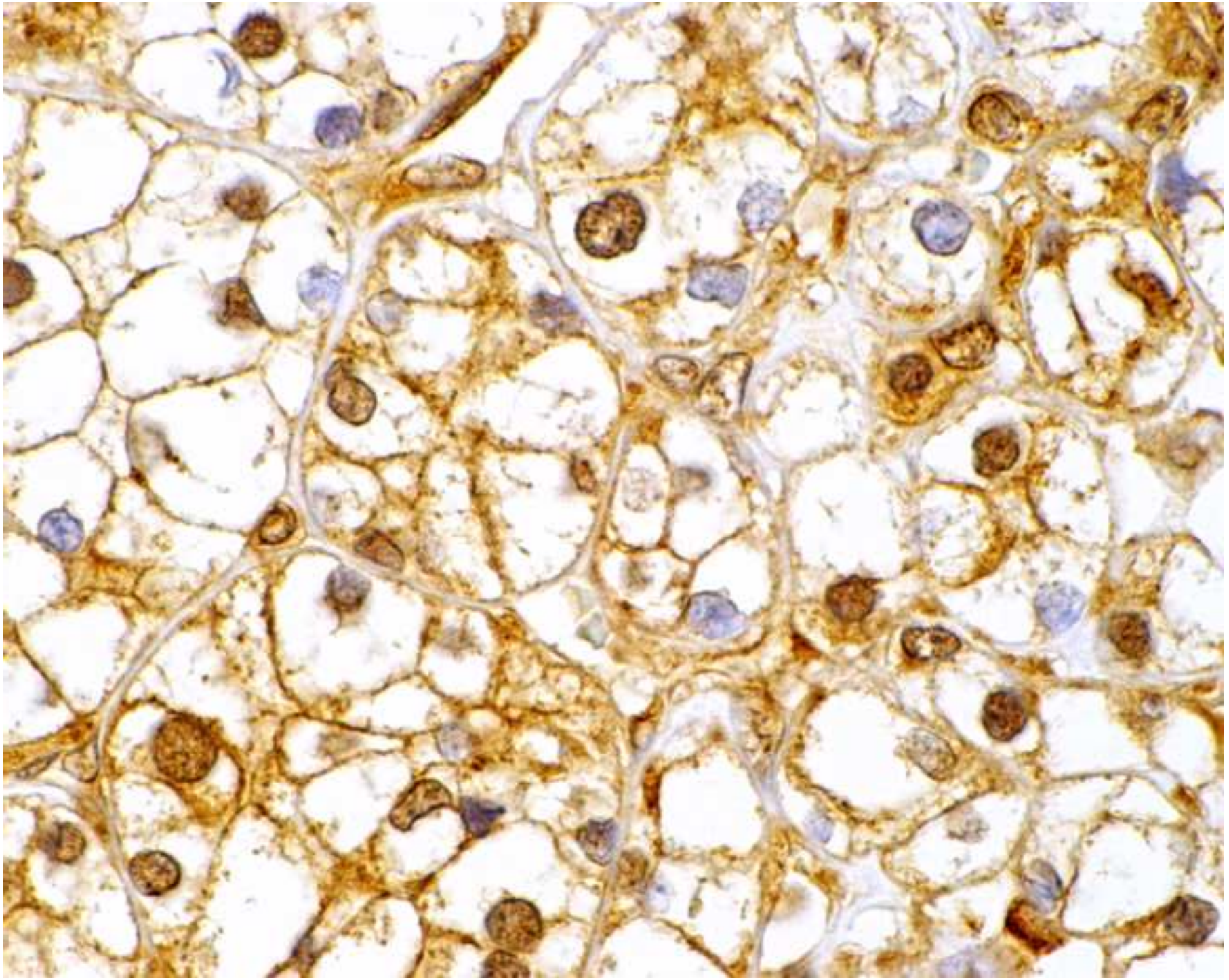

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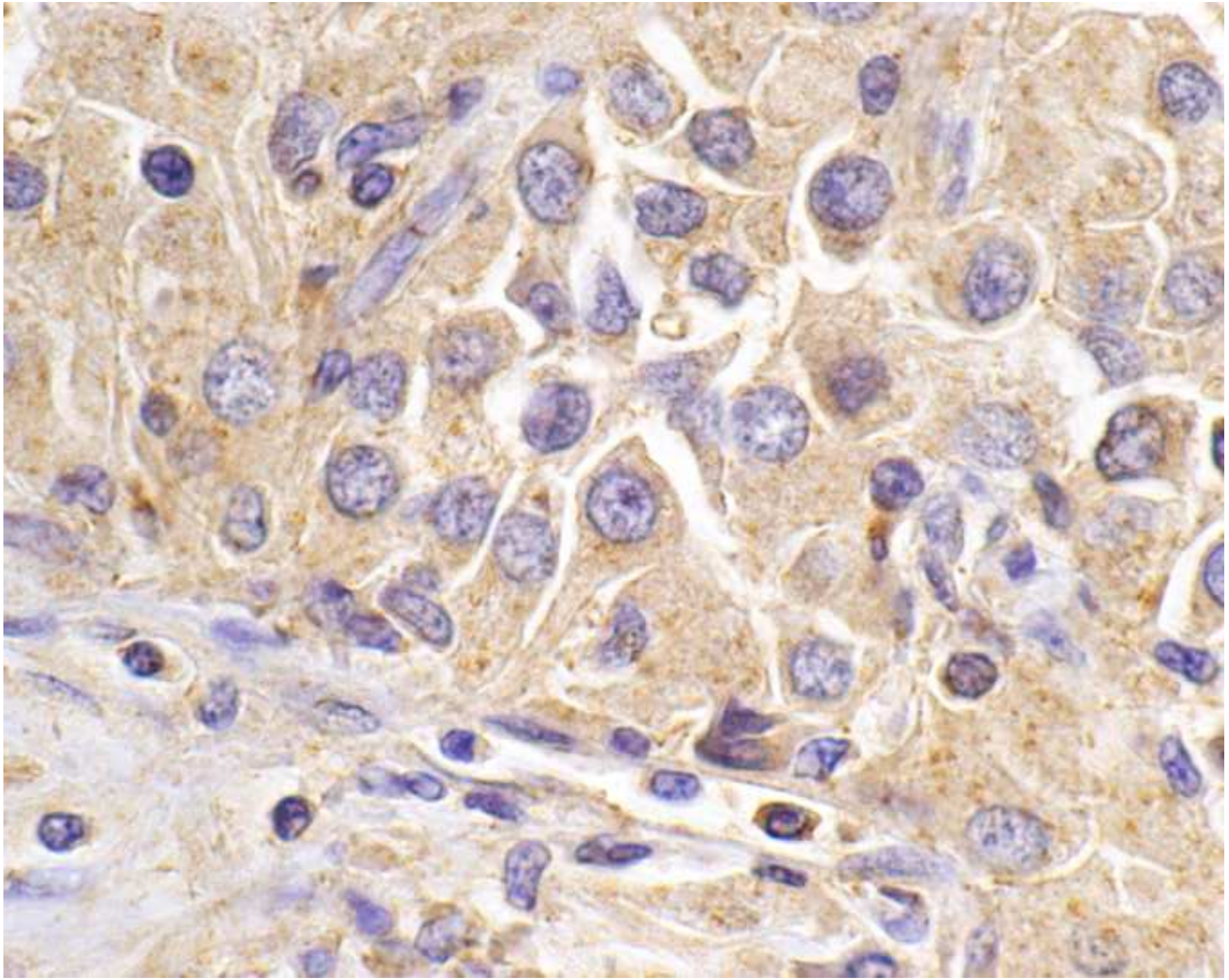

Figure 3a
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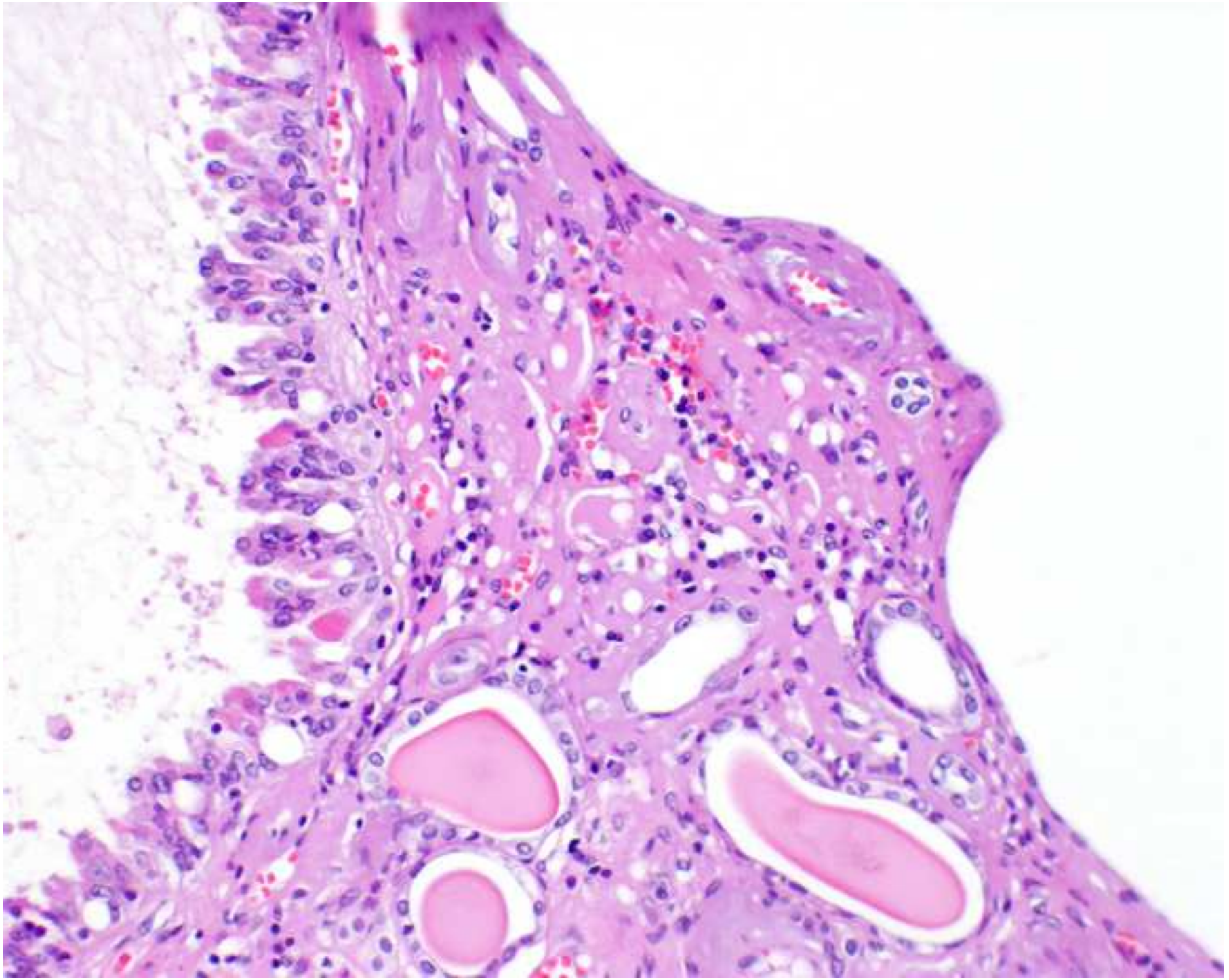

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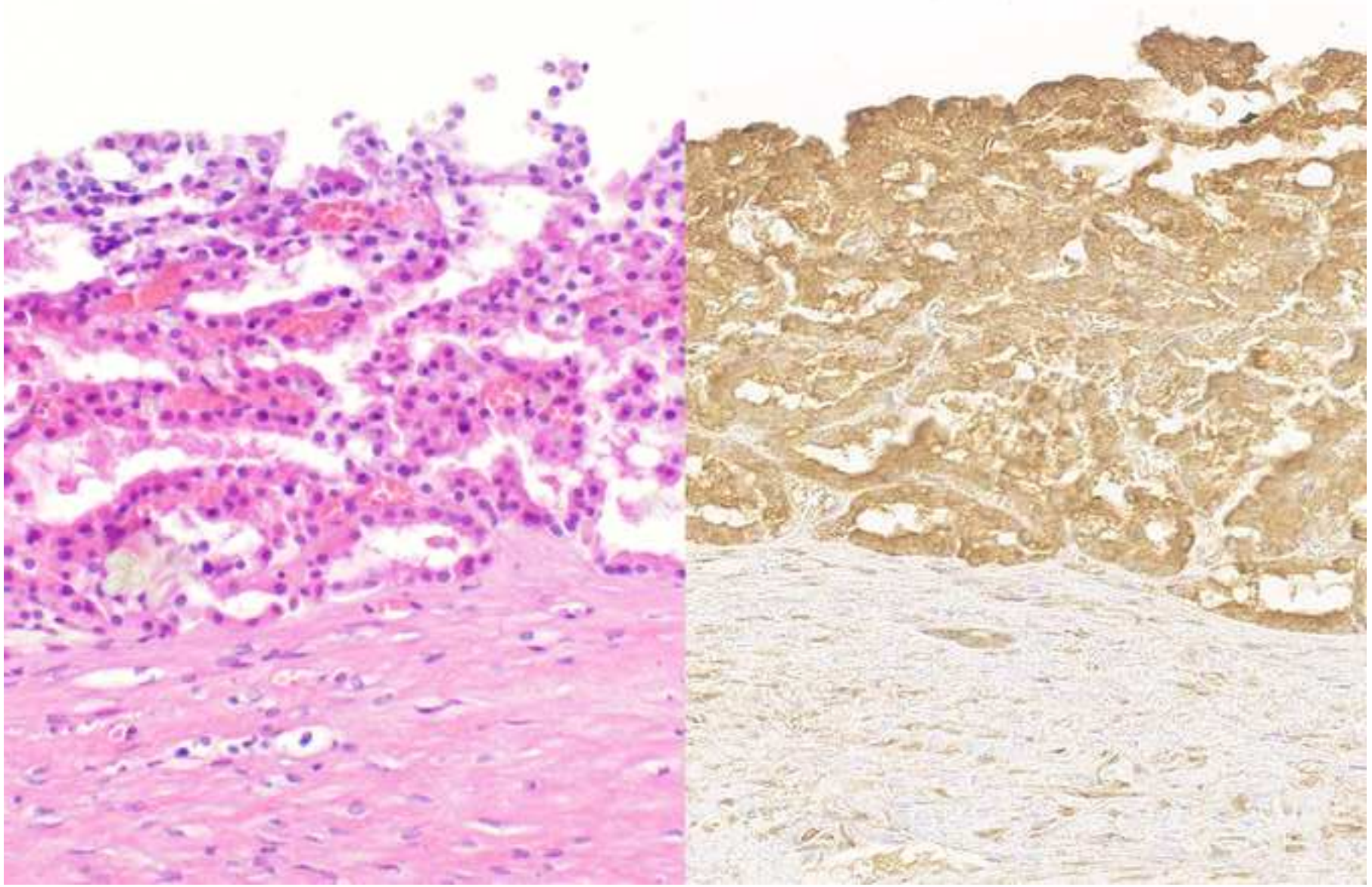

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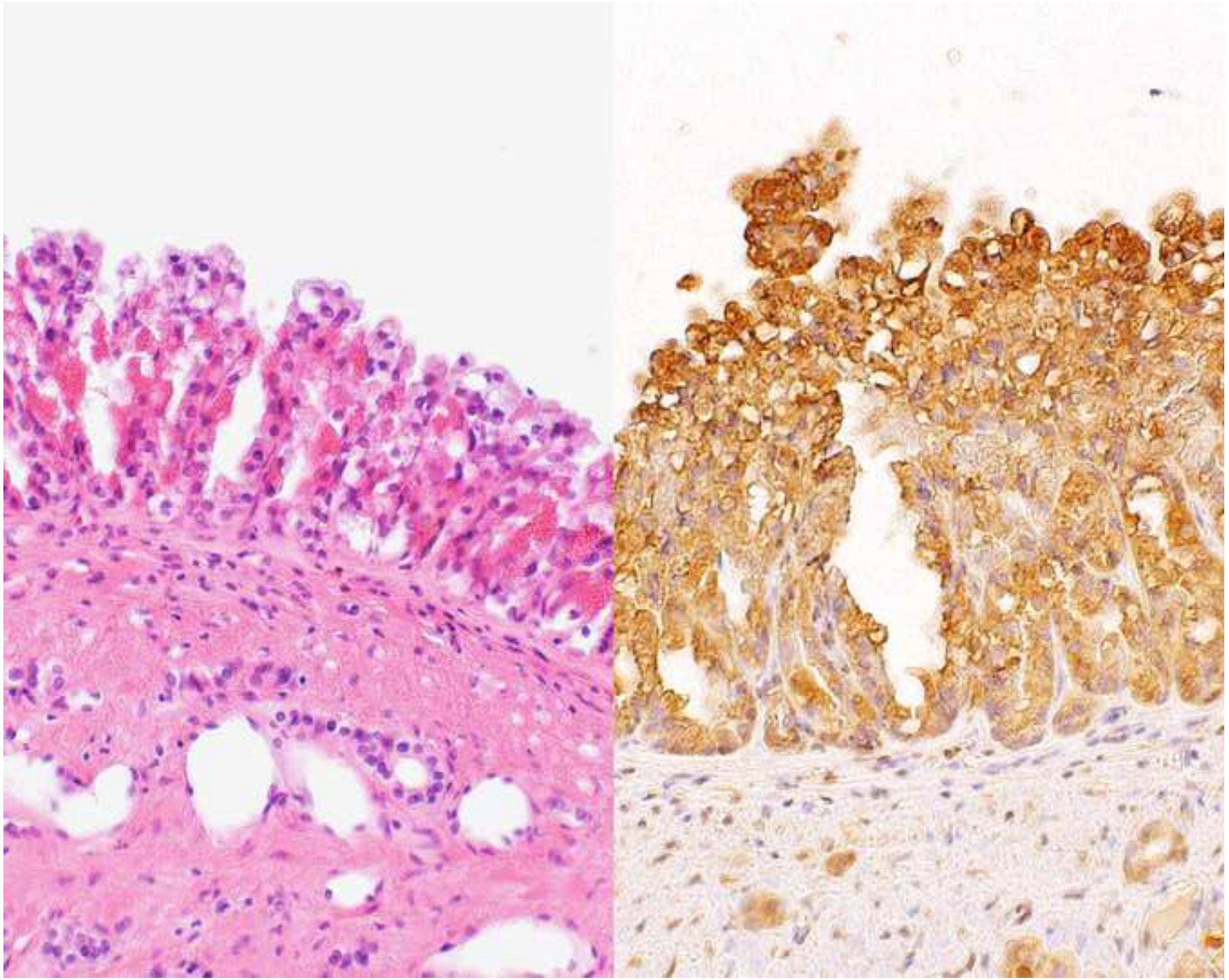

Figure 4a
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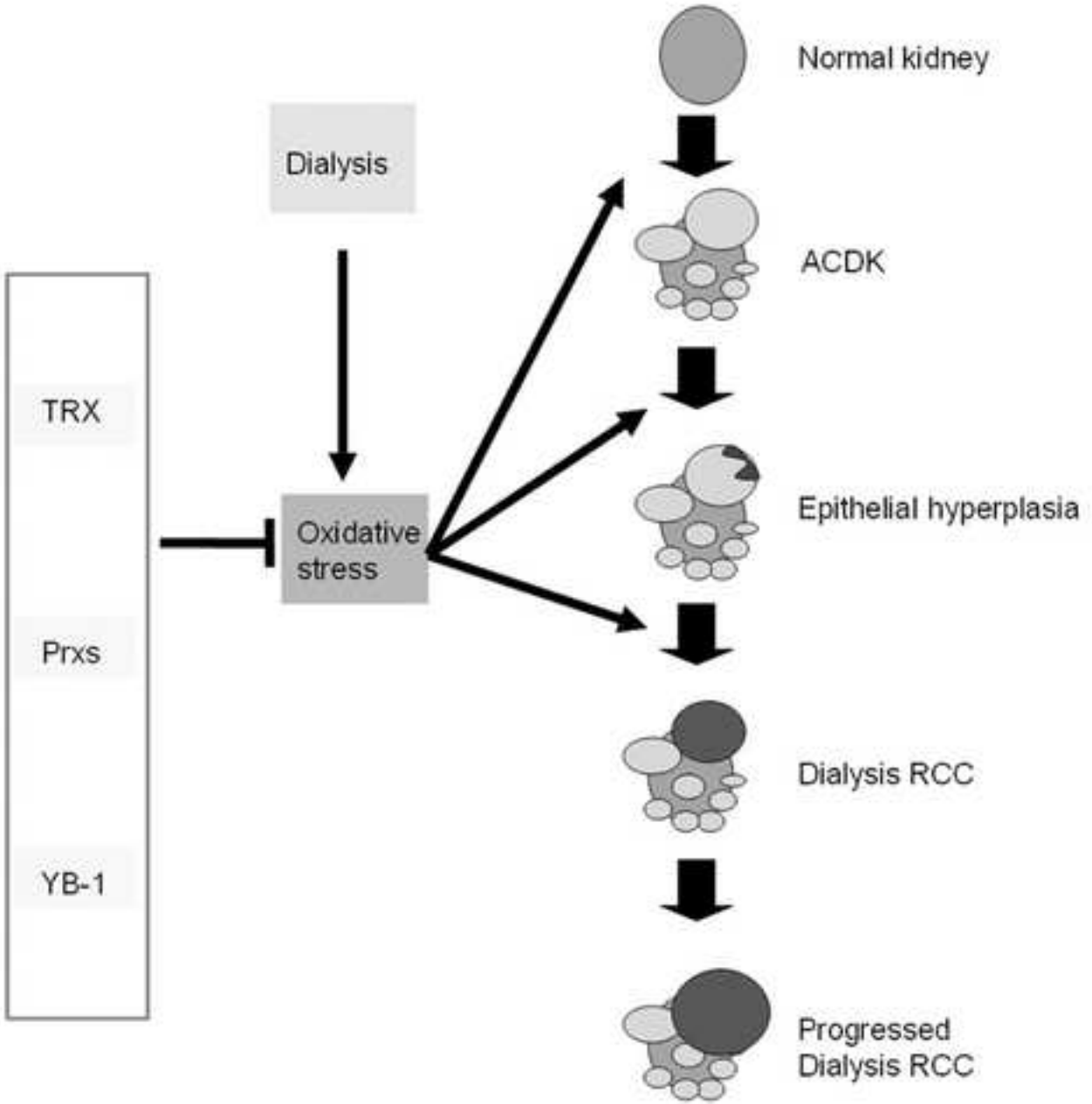

Figure 4b

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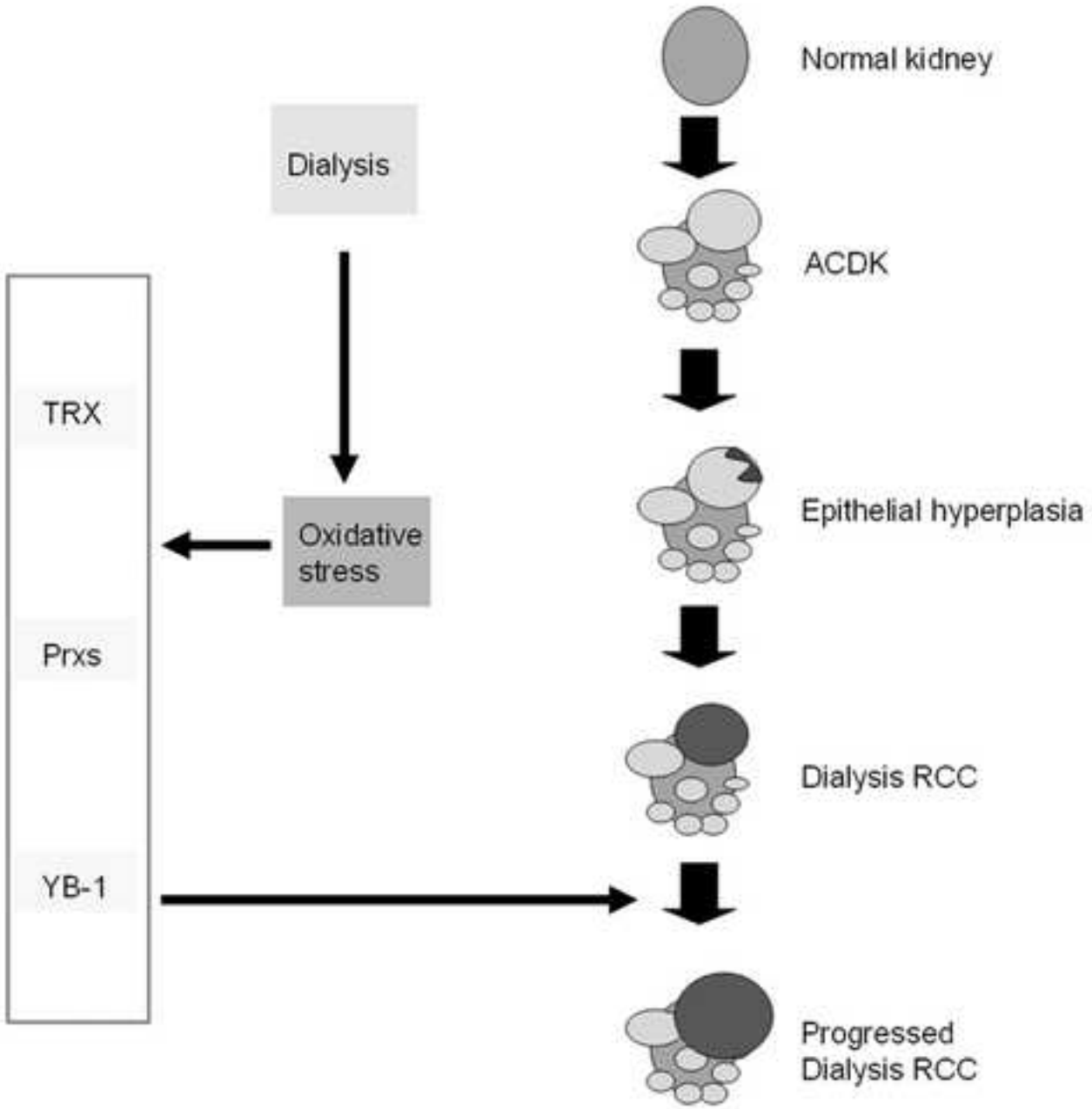

Table 1 Primary antibodies used in immunohistochemical study

Antibody	Manufacturer	Clone	Dilution	Pretreatment
Prx1	Abnova (Taipei, Taiwan)	4B11-G10	1:100	MW: 10min
Prx2	ProteinTech (Chicago, IL)	Rabbit Polyclonal	1:100	MW: 10min
Prx3	Thermo Fisher Scientific (Waltham, MA)	Rabbit Polyclonal	1:100	MW: 5min
Prx4	Santa Cruz (Santa Cruz, CA)	F-15	1:100	MW: 5min
Prx5	Prepared (Ref. 17)	Rabbit Polyclonal	1:100	MW: 10min
Prx6	Chemicon (Temecula, CA)	8H11	1:100	MW: 10min
TRX	Abcam (Cambridge, UK)	Rabbit polyclonal	1:6400	MW: 5min
YB-1	Prepared (Ref. 18)	Rabbit polyclonal	1:100	MW: 5min

Table 2 Clinicopathological features of 92 cases

	Sporadic RCC (n=49)	Dialysis RCC (n=43)	p Value
Mean age			
	60.9 (range, 37-83)	55.2 (range, 39-70)	0.032
Sex			
Male	40	31	ns
Female	9	12	
Histology (WHO(2004), Ref.16,21)			
Clear cell	39	30	ns
Papillary	5	10	
(Type 1)	(2)	(0)	
(Type 2)	(3)	(4)	
(Mixed)	(0)	(6)	
Chromophobe	2	1	
Multilocular cystic	2	0	
Purely sarcomatoid	1	0	
Unclassified	0	2	
Histology reported in ACDK (Ref.31)			
Clear cell		29	
Papillary		7	
Chromophobe		1	
ACD		5	
CCP		1	
Fuhrman grade			
>2	13	15	ns
≦2	36	28	
AJCC Stage			
T1	40	38	ns
T2	3	2	
T3	6	3	
Mean diameter (cm)			
	4.08 (range, 1.0-5.5)	3.28 (range, 1.0-10.2)	ns

ACDK: Acquired cystic disease of the kidney

ACD: Acquired cystic disease-associated RCC

CCP: Clear cell papillary RCC

ns: not significant

Table 3

Table 3 Results of immunohistochemistry

		Sporadic RCC (n=49)	Dialysis RCC (n=43)	Hyperplastic epithelium (n=17)
Prx1	(-)	14	5	1
	Low (+)	15	18	0
	High (+)	20	20	16
	Average score	3.8	4.9	6.9
Prx2	(-)	12	7	0
	Low (+)	16	18	0
	High (+)	21	18	17
	Average score	4.1	4.5	7.5
Prx3	(-)	22	12	0
	Low (+)	16	9	0
	High (+)	11	22	17
	Average score	3.8	5.4	7.7
Prx4	(-)	30	17	0
	Low (+)	11	8	0
	High (+)	8	18	17
	Average score	2.3	3.9	7.5
Prx5	(-)	35	17	1
	Low (+)	3	8	0
	High (+)	11	18	16
	Average score	1.8	3.4	7.1
Prx6	(-)	17	10	1
	Low (+)	9	14	0
	High (+)	23	19	16
	Average score	3.8	4.2	7.0
TRX	(-)	33	19	1
	Low (+)	8	11	0
	High (+)	8	13	16
	Average Score	2.3	3.1	6.8
YB-1	Average Score	12.8	39.2	39.0

Table 4　　P value of multiple comparison

	Dialysis RCC vs. Sporadic RCC	Hyperplastic epithelium vs. Sporadic RCC	Hyperplastic epithelium vs. Dialysis RCC
Prx1	ns	0.003	0.008
Prx2	ns	0.001	<0.001
Prx3	0.037	<0.001	0.009
Prx4	0.046	<0.001	0.001
Prx5	0.021	<0.001	0.005
Prx6	ns	0.012	0.008
TRX	ns	<0.001	<0.001
YB-1	<0.001	<0.001	ns

ns: not significant

Table 5 Immunohistochemical results with reference to the duration of dialysis

	<120 months (n=15)	>120 months (n=19)	Spearman's rho	p
Prx 1	4.2	5.7	0.43	0.010
Prx 2	4.3	5.2	0.30	ns
Prx 3	4.6	6.0	0.38	0.027
Prx 4	2.8	4.6	0.35	0.044
Prx 5	2.3	4.6	0.37	0.030
Prx 6	3.3	5.1	0.37	0.030
TRX	3.3	2.7	0.05	ns
YB-1	39.4	34.7	0.12	ns

ns: not significant

Table 6 Correlation between immunohistochemical results and clinicopathological factors

		Age	Sex	Histology	Fuhrman grade	AJCC stage	Diameter
Prx1	Sporadic RCC	ns	ns	<0.001	ns	ns	ns
	Dialysis RCC	ns	ns	<0.001	<0.001	ns	ns
Prx2	Sporadic RCC	ns	ns	<0.001	ns	ns	ns
	Dialysis RCC	ns	ns	<0.001	<0.001	ns	ns
Prx3	Sporadic RCC	ns	ns	<0.001	ns	ns	ns
	Dialysis RCC	ns	ns	<0.001	<0.001	ns	ns
Prx4	Sporadic RCC	ns	ns	<0.001	ns	ns	ns
	Dialysis RCC	ns	ns	<0.001	<0.001	ns	ns
Prx5	Sporadic RCC	ns	ns	<0.001	ns	ns	ns
	Dialysis RCC	ns	ns	<0.001	<0.001	ns	ns
Prx6	Sporadic RCC	ns	ns	<0.001	ns	ns	ns
	Dialysis RCC	ns	ns	<0.001	<0.001	ns	ns
TRX	Sporadic RCC	ns	ns	ns	ns	ns	ns
	Dialysis RCC	ns	ns	0.017	ns	ns	ns
YB-1	Sporadic RCC	ns	ns	ns	ns	ns	ns
	Dialysis RCC	ns	ns	ns	ns	ns	ns

ns: not significant

Peroxiredoxins, thioredoxin and Y-box-binding protein-1 expression in renal cell carcinoma arising in patients on dialysis

Fumiyoshi Fushimi,[1] Kenichi Taguchi,[2] Hiroto Izumi,[3] Kimitoshi Kohno,[3] Michihiko Kuwano,[4] Mayumi Ono,[4] Yutaka Nakashima,[5] Tetsuro Takesue,[5] Seiji Naito,[6] and Yoshinao Oda,[1]

[1]Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Higashi-ku, Fukuoka, Japan

[2]Department of Pathology, National Kyushu Cancer Center, Minami-ku, Fukuoka, Japan

[3]Department of Molecular Biology, University of Occupational and Environmental Health, Kitakyushu, Fukuoka, Japan

[4]Department of Pharmaceutical Oncology and Laboratory of Molecular Cancer Biology, Graduate School of Pharmaceutical Sciences, Kyushu University, Higashi-ku, Fukuoka, Japan

[5]Department of Pathology and Department of Urology, Fukuoka Red Cross Hospital, Minami-ku, Fukuoka, Japan

[6]Department of Urology, Graduate School of Medical Sciences, Kyushu University, Higashi-ku, Fukuoka, Japan

Corresponding author and requests for reprints: Yoshinao Oda, Department of Anatomic Pathology, Pathological Sciences, Graduate School of Medical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan

E-mail: oda@surgpath.med.kyushu-u.ac.jp

Tel: +81-92-642-6061, FAX: +81-92-642-5968

Abstract

Patients with end-stage renal disease are exposed to increased oxidative stress and impairment of antioxidant mechanisms. We focused on dialysis renal cell carcinoma (RCC), including epithelial hyperplasia in acquired cystic disease of the kidney (ACDK), and have attempted to obtain some insights into the carcinogenesis and tumor progression in terms of the cell's defense mechanism associated with oxidative stress by investigating the immunohistochemical expression of antioxidant proteins. We evaluated retrospectively a total of 43 cases of dialysis RCC and as a control group, 49 cases of sporadic RCC. Peroxiredoxin (Prx) 1,3,4,5,6 expression of dialysis RCC was positively correlated with the duration of dialysis. In epithelial hyperplasia in 17 cases of acquired cystic disease of the kidney, Prxs and thioredoxin were highly expressed. Moreover, Prx 3,4,5 immunoreactivity and nuclear expression of Y-box-binding protein-1 in dialysis RCC were at higher levels than those in sporadic RCC. In dialysis RCC, Prx 3,4,5 immunoreactivity was positively correlated with the Fuhrman nuclear grade. These data suggest that oxidative stress on dialysis could enhance antioxidant activity to prevent carcinogenesis. After carcinogenesis, antioxidant activity might create an advantageous phenotype to promote and progress dialysis RCC.

Key words: oxidative stress, peroxiredoxin, Y-box-binding protein-1, renal cell carcinoma, acquired cystic disease of kidney

Introduction

The association between dialysis patients and both increased oxidative stress and the impairment of the antioxidant defense mechanism is well-established [1]. The increased tendency of dialysis patients to develop renal cell carcinoma (RCC) compared to the general population is reported also, although its carcinogenesis remains poorly understood [2]. In a previous investigation, we evaluated oxidative stress by immunohistochemical expression of 8-oxoguanine and found a higher accumulation of 8-oxoguanine in dialysis RCC than in sporadic RCC [3]. This result suggests in dialysis RCC the level of oxidative stress may be correlated with carcinogenesis.

DNA oxidation may be an important factor in carcinogenesis. Oxidative stress is known to modulate cell proliferation and apoptosis, and induce synthesis of the growth factors that play an important role in tumor promotion and progression [4]. Antioxidant enzymes regulate the cellular redox state and constitute the major cellular protection against oxidative stress. Oxidant-antioxidant balance, therefore, may be important not only for carcinogenesis but also for tumor promotion and progression. Moreover, in most instances, dialysis RCC is considered to develop in association with acquired cystic disease of the kidney (ACDK) [5]. Cysts in ACDK are lined by flattened to cuboidal epithelium, and in most cases, focal epithelial hyperplastic proliferation can be seen. This epithelial hyperplasia arising in ACDK may pose an increased risk for the development of RCC [6]. In hyperplasia associated with dialysis and/or oxidative stress,

there may be a link between DNA damage and carcinogenesis.

Peroxiredoxin isoforms (Prxs) and thioredoxin (TRX) are ubiquitously distributed in all organisms including bacteria, plants and animals [7, 8]. Prxs and TRX are antioxidant enzymes that act as peroxidases, and have emerged as key molecules in the antioxidant defense mechanism. In recent years Prxs and TRX have been shown to have roles in cancer prevention, but they have diverse functions and reports link them both to the prevention and the promotion of the cancer and their exact role is still controversial [9-13].

Y-box-binding protein-1 (YB-1) is a transcription factor that plays important roles in cell proliferation, DNA replication and drug resistance. Cytoplasmic YB-1 is reported to translocate to the nucleus during UVA-induced oxidative stress [14]. YB-1 is also expected to act on recognition of oxidative DNA damage in the DNA repair pathway [15].

In this report we focused on dialysis RCC, including epithelial hyperplasia arising in ACDK. We attempted to obtain some insights into carcinogenesis and tumor progression in term of the cell's defense mechanism associated with oxidative stress by investigating immunohistochemical Prxs, TRX and YB-1 expression.

Materials and methods

Patients

A total of 43 cases of renal neoplasm in patients on dialysis between 1992 and 2008 were obtained from the Department of Anatomic Pathology, Kyushu University and from the Department of Pathology, Fukuoka Red Cross Hospital. As a control group, 49 cases of sporadic RCC were selected. Because of specimen deterioration, the number of cases decreased to 38 cases of dialysis RCC and 49 cases of sporadic RCC. Five cases of dialysis RCC were newly obtained from the Department of Anatomic Pathology of Kyushu University between 2006 and 2008. The five new cases were diagnosed as described in our previous report [3]. RCCs were classified according to the 1997 UICC/ AJCC consensus and WHO (2004) classification systems [16]. There was no family history of renal neoplasm in all 92 cases. The institutional review board at Kyushu University approved this study (permission code: 24-59).

Immunohistochemistry

Immunohistochemical staining was performed using 10% formalin-fixed, paraffin-embedded tissue sections using the avidin–biotin–peroxidase method, with positive and negative controls. Details of the primary antibodies are summarized in Table 1. Anti-Prx5 [17] and anti-YB-1 [18] antibodies were prepared as previously described.

Interpretation and scoring of immunohistochemical preparations

Immunoreactivity was interpreted as positive based on the presence of cytoplasmic staining for Peroxiredoxin isoforms (Prxs) and Thioredoxin (TRX). A proportion score was assigned to represent the estimated proportion of positively stained tumor cells (0 = none, 1 = <1/100, 2 = 1/100 to 1/10, 3 = 1/10 to 1/3, 4 = 1/3-2/3, 5 = >2/3). The average estimated intensity of staining in positive cells was given as an intensity score (0 = none, 1 = weak, 2 = intermediate, 3 = strong). The proportion score and the intensity score were added to get a total score. The immunohistochemical expression status was trichotomized based on the total score, according to our previously report: negative (-), 0; low positive (low +), 2 to 4; and high positive (high +); 5 to 8 [19]. Nuclear immunohistochemical expression of YB-1 protein was evaluated as a percentage of positively stained nuclei in a selected hot spot [20]. Two pathologists (FF, KT) evaluated the immunohistochemistry to insure accuracy of interpretation.

Statistical analysis

The clinicopathological factors were analyzed using the chi-square test or unpaired student's t-test. The Yates Chi-square test was used to assess the significance of the differences in the immunoreactivity between the expressions of Prxs and TRX in dialysis RCC, sporadic RCC and hyperplastic epithelium in ACDK. The unpaired t-test

was used to assess the significance of the differences between the nuclear immunohistochemical expression of YB-1 in dialysis RCC, sporadic RCC and hyperplastic epithelium in ACDK. The Spearman rank method was used to calculate correlation coefficients between duration of dialysis and immunohistochemical results in dialysis RCC. P values less than .05 were considered statistically significant.

Results

Clinicopathological findings

The clinicopathological data on 43 cases of dialysis RCC and 49 cases of sporadic RCC are summarized in Table 2. The 92 patients with renal neoplasm analyzed in this study had an age range of 37-83 years. Histologically, the frequency of papillary type was higher in dialysis RCC (10/43 cases: 23.3%) than in sporadic RCC (5/49 cases: 10.2%), but was not statistically significant (p=0.159). In dialysis RCC, however, clear-cut classification is difficult in many cases. In particular, type 1 and type 2 papillary RCC were not readily distinguished in histology. The tumors that morphologically did not fit the criteria for AJCC consensus and WHO (2004) classification systems were designated mixed type papillary RCC in reference to Yang's paper [21]. There was no definite type 1 papillary RCC in dialysis RCC. The other clinicopathological findings are almost the same as described in our previous report [3].

Immunohistochemical expression of Prxs, TRX and YB-1 in sporadic and dialysis RCC

In non-neoplastic renal tissue in both sporadic and dialysis RCC, all Prxs and TRX showed varying immunoreactivity in the cytoplasm of proximal or distal renal tubules. Cells of collecting ducts and glomerular epithelial cells showed no immunoreactivity whatsoever. Prxs and TRX immunoreactivity in RCC were shown in the tumor cytoplasm, regardless of isoforms (Figure 1a, 1b). The results of immunohistochemical study for Prxs, TRX and YB-1 are summarized in Table 3. All Prxs and TRX immunoreactivity in dialysis RCC have higher scores than in sporadic RCC. In particular, expressions of Prx3($p=0.037$), Prx4($p=0.046$), Prx5($p=0.021$) were significantly higher in dialysis RCC (Table 4).

YB-1 protein expression was observed in both cytoplasm and nucleus or only in cytoplasm in renal cell carcinoma (Figure 2). Dialysis RCC showed statistically higher nuclear immunohistochemical expression of YB-1(39.2%, mean), compared to the sporadic RCC (12.8%, mean) (Table 4, $p<0.001$).

The relationship between the duration of dialysis and immunohistochemical expression in dialysis RCC

The mean duration of dialysis was 146 months in patients with dialysis RCC who had an accurate dialysis record (n=34). The mean duration of dialysis was 16

months in patients with no cysts (n=6), 174 months in those with acquired cystic disease (n=28) and was statistically different ($p<0.001$). Immunohistochemical results with reference to the duration of dialysis for over 120 months and under 120 months are summarized in Table 5. The mean total scores of all Prxs were higher in patients with a long duration of dialysis (>120 months). Moreover, positive correlations between the duration of dialysis and Prx 1,3,4,5,6 expression were observed in dialysis RCC, as evaluated by Spearman's rank method. In contrast, Prx2 expression, TRX expression and nuclear expression of YB-1 were not correlated with duration of dialysis.

Prxs TRX and YB-1 immunoreactivity and its correlation with clinicopathological factors

Table 6 shows the correlations between Prxs, TRX and YB-1 immunoreactivity, and clinicopathological data in sporadic and dialysis RCC. The clear cell type RCC and papillary type RCC were compared. The papillary type showed higher immunoreactivity of Prxs compared to the clear cell type in both dialysis RCC and sporadic RCC ($p<0.001$). In dialysis RCC, there was no definite difference between type 2 and mixed type papillary RCC in terms of Prxs, TRX and YB-1 immunoreactivity. In dialysis RCC, the immunoreactivity of all the six Prxs was positively correlated with the Fuhrman nuclear grade, which is one of the major prognostic features of RCC. On the other hand, in sporadic RCC, no correlation was

observed between Prxs immunoreactivity and the Fuhrman nuclear grade. The other clinicopathological factors such as stage and tumor diameter had no correlation with Prxs immunoreactivity. No significant correlation was observed between nuclear expression of YB-1 and clinicopathological factors.

Epithelial hyperplasia in acquired cystic disease of kidney

In 43 Dialysis RCC, at least 29 were accompanied by acquired cystic disease of the kidney (ACDK). Epithelial hyperplasia was detected in 17/29 cases of ACDK (Figure 3). Epithelium that lines the ACDK is mostly flattened, and hyperplastic epithelium is irregularly distributed. The results of immunohistochemical study for Prxs, TRX and YB-1 in epithelial hyperplasia in ACDK are summarized in Table 3. In all 29 cases of ACDK, the lining flat epithelial cells show variable immunoreactivity of Prxs and TRX from negative to high positive.

On the other hand, in epithelial hyperplasia in 17 cases of ACDK, Prxs and TRX immunoreactivity was high except in one case in which Prx1,5,6 and TRX expression was not observed. All Prxs and TRX immunoreactivity in epithelial hyperplasia of ACDK had significantly higher scores compared with those in both sporadic RCC and dialysis RCC (Table 3, Table 4). The mean nuclear expression of YB-1 in epithelial hyperplasia in ACDK was 39.0%, compared with 12.8% in sporadic RCC and was statistically different ($p<0.001$).

Discussion

Several characteristic genetic alterations have been found to be associated with the carcinogenesis of renal cell carcinoma (RCC), such as mutations of the VHL gene. To date, however, there is little evidence of specific genetic mutation in patients on dialysis, although a few reports show mutations of the VHL gene [22]. It is reported that the genetics of renal cell carcinoma associated with acquired cystic renal disease may differ from those occurring in the general population [2]. The histological types of dialysis RCC is also distinct from that of sporadic RCC, especially in patients with a duration of dialysis for more than 120 months [23]. Clear cell type RCC was the predominant histological type in patients with a duration of dialysis of less than 120 months, while the predominance of clear cell RCC decreased in those on dialysis for more than 120 months. The risk of RCC is considered to be higher in patients with a longer duration of dialysis. Several pathological mechanisms could contribute to this higher risk, including the increased synthesis of reactive oxygen species. Dialysis induces oxidative stress and may be associated with oxidative DNA damage and carcinogenesis. We hypothesized that oxidative stress induces carcinogenesis in dialysis patients, and found significantly higher levels of 8-oxoguanine in dialysis RCC than in sporadic RCC [3]. The process of carcinogenesis by oxidative stress is accompanied by

cumulative mutations in genetic pathways that are advantageous to carcinogenesis. The carcinogenesis process by oxidative stress, therefore, is thought to involve many genes and is a result of multistage mutagenesis. Oxidative stresses have a complex nature and oxidant-antioxidant balance might be important for carcinogenesis and tumor progression. The expression of antioxidant enzyme or stress protein in response to oxidative stress is to be expected.

Prxs and TRX are antioxidant enzymes and they also modulate intracellular signaling pathways related to apoptosis and cell proliferation. YB-1 is reported to be a transcriptional regulatory factor for several genes including the multidrug resistance 1 gene in human malignancy [24]. Although, to the best of our knowledge, there are no reports that YB-1 directly regulates antioxidant enzymes, such as Prxs and TRX, the association between YB-1 and oxidative stress has been reported [14, 15]. In the present study, Prx 1,3,4,5,6 expression of dialysis RCC was positively related with the duration of dialysis. This result suggests that the longer duration of dialysis causes the higher oxidative stress and antioxidative activities. ACDK bears a strong relationship to the duration of dialysis, and it is reported as being present in more than 90% of patients at 120 months or more of dialysis [25]. Epithelial hyperplasia arising in ACDK is a characteristic feature of kidneys on dialysis and has been implicated in the pathogenesis of renal cell carcinoma [6]. Our results showed Prxs and TRX immunoreactivity and nuclear expression of YB-1 in epithelial hyperplasia in ACDK were at high levels.

Moreover, Prx 3,4,5 immunoreactivity and nuclear expression of YB-1 in dialysis RCC were at higher levels compared with those in sporadic RCC. Therefore, expression of Prx 3,4,5 and YB-1 in epithelial hyperplasia in ACDK and dialysis RCC may be a response to the stimuli with oxidative stress. In dialysis RCC, all the six Prxs' immunoreactivity were positively correlated with the Fuhrman nuclear grade, which is defined as four nuclear grades in the order of increasing nuclear size, irregularity and nucleolar prominence [26]. The Fuhrman nuclear grade is one of the important prognostic features of RCC [27]. Our data therefore suggest that Prxs, TRX and YB-1 may have effects of the promotion and progression of dialysis RCC. In Japan, patients with dialysis have been closely monitored for dialysis RCC. Early detection is expected from the fact that in most cases the AJCC stage is T1 and the diameter of most dialysis RCC tumors is less than 4cm. It is speculated that AJCC stage and tumor diameter are not useful prognostic features and the Fuhrman nuclear grade may be the most useful prognostic feature in the case of dialysis RCC in Japan.

Our results also suggest the bipolar roles of antioxidant defense mechanisms in carcinogenesis and tumor progression (Figure 4). Prxs, TRX and YB-1 in dialysis RCC may not only be an indicator of oxidative stress, but may also represent an adaptive response by tumor cells to adjust and survive the oxidative environment. Since Prxs, TRX and YB-1 are highly expressed in epithelial hyperplasia in ACDK, it is expected that antioxidants are associated with anti-cancerous effects in the early

development of the carcinogenesis (Figure 4a). But once carcinogenesis occurs, constant oxidative stress may work to aid tumor protection in maintaining the expression of antioxidant proteins, such as Prxs, TRX and YB-1, compared to the general population (Figure 4b). Prxs are reported to play a role in cell proliferation, differentiation, immune response, protection of oxidant-sensitive proteins, regulation of cellular hydrogen peroxide and control of apoptosis, processes involving in oxidative stress-related cellular signaling [28]. Our results suggest that constant oxidative stress, such as dialysis, could enhance antioxidant activity to prevent carcinogenesis, but after carcinogenesis, antioxidant activity leads to the acquisition of advantageous phenotype which promotes and progresses the cancer. The functions of Prxs include not only the detoxification of peroxide, but also the increase of cell survival and proliferation. Protection against apoptotic stimuli is one possible explanation of the increased cell survival and proliferation. Our results show that Prx 3,4,5 especially may play a very specific role in the carcinogenesis and tumor progression. It is reported that the expression of peroxiredoxins, especially Prx 3,4,5 are increased in breast malignancy, in accordance with our results [29]. However, the significance of specific expression of Prx isoforms remains unresolved and further research is needed. It is reported that Prxs could be induced by irradiation, which also induce oxidative stress [30]. Its expression can be taken as an adaptive response and could be associated with the resistance of tumor cells to oxidant-generating irradiation. The nuclear expression of YB-1 is

reported to be associated with poor prognosis in cancer patients. Since YB-1 is a regulatory factor for multidrug resistance 1 gene, it is considered likely that YB-1 is responsible for treatment failure in cancer patients. Our study joins others in highlighting the complex nature of adaptive response in relation to carcinogenesis.

It is reported that RCC arising in a background of ACDK shows morphologic features that are not seen in sporadic RCC. We have also classified histological subtypes of dialysis related RCC based on Tickoo's paper [31] (Table 2). Two types of RCC were newly designated; acquired cystic disease-associated RCC (ACD-associated RCC) in five cases and clear cell papillary RCC in one case. ACD-associated RCC is characterized by eosinophilic cytoplasm with Fuhrman's grade 3 nuclei, frequent association with intratumoral oxalate crystals, and various combinations of acinar, solid alveolar, solid sheet-like, micro-cystic, macro-cystic, and papillary architecture. Prominent papillary architecture in our cases led us to classify them as papillary RCC. Various combinations of architecture led us to interpret them as RCC, unclassified. All Prxs and TRX immunoreactivity in 5 cases of ACD-associated RCC were high and the mean nuclear expression of YB-1 was 30%. ACD-associated RCC showed immune profiles similar to epithelial hyperplasia in ACDK. This may imply that epithelial hyperplasia in ACDK is putative precursor lesion of ACD-associated RCC.

In conclusion, we analyzed dialysis RCC, including epithelial hyperplasia arising in ACDK, by investigating immunohistochemical Prxs, TRX and YB-1

expression. All Prxs, TRX and YB-1 expression in dialysis RCC, including epithelial hyperplasia arising in ACDK, are at higher levels compared with that in sporadic RCC. In dialysis RCC, Prx 3,4,5 immunoreactivity was positively correlated with the Fuhrman nuclear grade, which is one of the major prognostic features of RCC. The findings of this study suggest that oxidative stress on dialysis could enhance antioxidant activity to prevent carcinogenesis. After carcinogenesis, antioxidant activity might create an advantageous phenotype to promote and progress dialysis RCC.

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Disclosure/conflict of interest

None.

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Figure legends

Figure 1

Immunohistochemical expression of Peroxiredoxin 3 and Thioredoxin in dialysis RCC.

(a) Clear cell RCC of a 59-year-old man shows strong cytoplasmic immunoreactivity for Peroxiredoxin 3 (score 8). (b) Papillary RCC of a 68-year-old female shows strong cytoplasmic immunoreactivity for Thioredoxin (score 7).

Figure 2

(a) Nuclear staining and (b) cytoplasmic staining of YB-1 protein**.**

Figure 3

Epithelial hyperplasia in acquired cystic disease of kidney and immunohistochemical expression of Peroxiredoxin 3 and Peroxiredoxin 5

(a) The cysts in acquired cystic disease of the kidney of a 70-year-old female are lined by flattened epithelium in the right and hyperplastic papillary epithelium in the left.

(b) Hyperplastic epithelium in acquired cystic disease of the kidney of a 53-year-old female shows strong cytoplasmic immunoreactivity for Peroxiredoxin 3 (score 8).

(c) Hyperplastic epithelium in acquired cystic disease of the kidney of a 44-year-old male shows strong cytoplasmic immunoreactivity for Peroxiredoxin 5 (score 8).

Figure 4

Potential mechanisms of antioxidant activity in the development of dialysis RCC

(a) Dialysis induces oxidative stress. Constant oxidative stress can result in cumulative DNA damage, which may be prevented by antioxidant proteins, such as Prxs.

(b) Constant oxidative stress induces the expression of antioxidant proteins, which can support the survival and growth of cancer cells.