



High sulfite oxidase expression could predict postoperative biochemical recurrence in patients with prostate cancer

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Abstract

Sulfite oxidase (SUOX) is a metalloenzyme that plays a role in ATP synthesis via oxidative phosphorylation in mitochondria and has been reported to also be involved in the invasion and differentiation capacities of tumor cells. Here, we performed a clinicopathological investigation of SUOX expression in prostate cancer and discussed the usefulness of SUOX expression as a predictor of biochemical recurrence following surgical treatment in prostate cancer. This study was conducted using Tissue Micro Array specimens obtained from 97 patients who underwent radical prostatectomy at our hospital between 2007 and 2011. SUOX staining was used to evaluate cytoplasmic SUOX expression. In the high-expression group, the early biochemical recurrence was significantly more frequent than in the low-expression group ($p = 0.0008$). In multivariate analysis, high SUOX expression was found to serve as an independent prognostic factor of biochemical recurrence (hazard ratio = 2.33, 95% confidence interval = 1.32–4.15, $p = 0.0037$). In addition, Ki-67-labeling indices were significantly higher in the high-expression group than in the low-expression group ($p = 0.0058$). Therefore, SUOX expression may be a powerful prognostic biomarker for decision-making in postoperative follow-up after total prostatectomy and with regard to the need for relief treatment.

Keywords Prostate cancer · SUOX · Biomarker · Biochemical recurrence · Oxidative phosphorylation

Introduction

Prostate cancer is the most common solid cancer and the second most frequent cause of death of men in the United States of America. In Japan, the widespread use of prostate-specific antigen (PSA) testing led to prostate cancer becoming the most prevalent cancer among men in 2016 [1]. Most patients have organ-confined or locally advanced tumors at diagnosis, and prostate cancer is generally considered to progress relatively slowly. Therefore, when surgery is selected

for the treatment of localized prostate cancer, the prognosis is generally good [2]. However, the rates of postoperative biochemical recurrence are 16–31% and 25–53% after 5 and 10 years, respectively [3, 4]. Some of these cases develop into castration-resistant prostate cancer after clinical recurrence, often leading to poor outcomes. Thus, biochemical recurrence is often used to justify the application of salvage therapies, such as endocrine therapy and radiotherapy.

Although recurrence of prostate cancer after radical treatment is generally assessed based on increase in PSA (biochemical recurrence) [5–7], studies have identified a variety of predictive factors for biochemical recurrence. Significant factors include a designation of “poor risk” in the D’Amico classification, positive surgical margins (RM1), preoperative PSA score, Gleason score at prostatectomy, and pathological staging. Of these, positive surgical margins (RM1) are the most important predictive factor for biochemical recurrence (recurrence rate: 1.5–6.0×) [8–16]. However, there are a few reports of effective biomarkers that can be used to predict biochemical recurrence.

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Sulfite oxidase (SUOX), a metalloenzyme found in mitochondria, interacts with molybdenum and heme as a coenzyme [17, 18]. SUOX is a cytochrome *b5* enzyme that belongs to the oxotransferase superfamily, which includes dimethyl sulfoxide reductase, xanthine oxidase, and nitrite reductase. SUOX converts electrons generated as a result of oxidation of sulfurous acid into sulfuric acid via cytochrome *c* and facilitates the transport of electrons used in ATP synthesis via oxidative phosphorylation; therefore, SUOX has been identified as one of several indicator molecules with respect to oxidative phosphorylation in ATP synthesis [19–21]. Although numerous research papers concerning SUOX deficiency have been published to date, SUOX deficiency has also been described in reports as a factor involved in the invasion and differentiation capacity of cancer cells [22]. In tongue cancer in particular, SUOX has been shown to be related to oncogenesis based on the results of comprehensive analysis of mRNA extracted from healthy and dysplastic mucous membranes as well as sites of cancerous invasion. However, a few studies have examined SUOX expression in cancer cases, and SUOX expression profiles in tumor cells have not been elucidated [23].

Accordingly, in this study, we evaluated SUOX expression in patients with biologically localized prostate cancer who had undergone surgical resection and investigated the feasibility of using serum PSA value, in conjunction with Gleason score and various histopathological factors, as a predictive factor of biochemical recurrence.

Materials and methods

Patients and tissue samples

Ninety-seven patients who underwent prostatectomy at Kurume University Hospital (Kurume, Japan) between January 2007 and December 2011 were enrolled in this study. As part of this study, the pathological diagnoses of the patients were re-examined. The following patients were excluded from this study beforehand: (1) patients who had undergone hormonal therapy and/or radiotherapy before surgery, (2) patients found to be at stage pT0 during surgery, and (3) patients from whom specimens could not be created using tissue microarray (TMA). All patients were pathologically diagnosed with prostatic adenocarcinoma. Paraffin-embedded samples of primary prostate cancer tissue from 97 patients were used to construct a TMA containing 22 cores per slide (two primary tissue cores per patient). Histopathological evaluations were performed by three pathologists (H.K., Y.N., and R.K.). Pathological diagnosis was performed according to the 2016 World Health Organization Classification of Tumors of the Urinary System and Male Genital Organs [24].

This study was approved by the Research Ethics Committee of Kurume University (#18161), which conforms to the guidelines of the Declaration of Helsinki.

Immunohistochemical analysis

Paraffin-embedded tissue samples were cut to a thickness of 4 μm , examined on coated slide glass, and labeled with anti-SUOX antibodies (1:600; Abcam, Cambridge, MA, USA) and anti-Ki-67 antibodies (NCL-Ki67-MM1; dilution 1:200; Leica Biosystems, Nussloch, Germany) using a BenchMark ULTRA (Ventana Automated Systems, Inc., Tucson, AZ, USA). Briefly, the slides were heat treated using Ventana's ULTRA cell conditioning 1 retrieval solution (CC1; Ventana Automated Systems, Inc.) for 36 min at 95 °C and incubated with anti-SUOX antibodies for 32 min at 37 °C. An automated system with a Ventana UltraVIEW 3,3'-diaminobenzidine (DAB) detection kit was used, including horseradish peroxidase multimer as the secondary antibody and DAB as the chromogen. Slides were incubated with secondary antibody for 30 min at 37 °C. For quantification of staining with anti-SUOX antibodies, the intensity score (scale 0–2: score 0; no expression, score 1; low expression, score 2; high expression) and population score (scale 0–5: score 0; 0, score 1; 0 ~ 1/100, score 2; 1/100 ~ 1/10, score 3; 1/10 ~ 1/3, score 4; 1/3 ~ 2/3, score 5; 2/3 ~) were added to generate a Histology Score (*H* score) with $H = \text{intensity} + \text{population score}$ (0–7). All immunohistochemical analyses were evaluated by two experienced pathologists who were unaware of the patients' clinical conditions. The scores of the two pathologists were added, and each patient was given a total expression score consisting of 15 levels from 0 to 14. We considered only cytoplasmic expression of SUOX as positive. Ki-67-labeling index (LI) was calculated as the percentage of tumor cells that showed positive expression. Furthermore, double staining using anti-SUOX and Ki-67 antibodies was performed in this study to assess the correlation between SUOX expression and Ki-67 LI. Resected prostate cancer tissue samples were labeled with Ki-67 and SUOX antibodies using the BenchMark ULTRA and Bond-III autostainer for double-staining analysis. BenchMark ULTRA was used for Ki-67. Briefly, the slide was heat-treated using Ventana's CC1 retrieval solution for 64 min, and incubated with Ki-67 antibody for 30 min. This automated system used the streptavidin–biotin complex method with DAB. Immunostaining with SUOX was performed on the same fully automated Bond-III system, and incubated with the SUOX antibody for 30 min. The automated system used a Bond Polymer Refine Red Detection Kit with Fast Red as the substrate chromogen. Counterstaining was performed with hematoxylin. Finally, nuclear Ki-67 was labeled brown with DAB, and cytoplasmic SUOX was labeled red with Fast Red.

Statistical analysis

We examined the correlations between SUOX expression and clinicopathological characteristics, such as age at diagnosis, serum PSA level at diagnosis, Gleason score at radical prostatectomy, pathological *T* stage, lymphatic invasion, peripheral nerve invasion, positive surgical margins, Ki-67 LI, and biochemical recurrence using Chi-square tests or Fisher's exact tests. The cut-off value of SUOX was determined by receiver-operating characteristic curve analysis. Cancer survival analysis was performed using the Kaplan–Meier method, log-rank test, and Cox's proportional hazards model. The threshold for statistical significance was set at $p < 0.05$. Biochemical recurrence was defined as an increase in PSA level over 0.2 ng/mL after two different measurements at least 3 months apart. The statistical software used was JMP Pro 13 (SAS Institute Inc., Cary, NC, USA).

Results

Patient's characteristics

The clinicopathological characteristics of the 97 patients are summarized in Table 1. The median postoperative follow-up period was 60 months (quartile, 46–86 months). The median age was 68 years (range 56–77 years), and the median PSA level at the initial diagnosis was 8.00 ng/mL (range 2.13–52.65). D'Amico risk stratification was low in 18 patients (18.6%), intermediate in 40 patients (41.2%), and high in 39 patients (40.2%). Gleason scores at prostatectomy were less than or equal to 6 in 15 patients (15.5%), equal to 7 (3 + 4) in 35 patients (36.1%), equal to 7 (4 + 3) in 33 patients (34.0%), and greater than or equal to 8 in 14 patients (14.4%). Pathological stages were T2 in 69 patients (71.1%), T3a in 21 patients (21.7%), and T3b in seven patients (7.2%). The numbers of patients with lymphatic and peripheral nerve invasion were 3 (3.1%) and 48 (49.5%), respectively. In addition, the number of patients with positive resection margins was 47 (48.5%). Overall, 53 patients (54.3%) experienced biochemical recurrence.

Immunohistochemical analysis of SUOX expression

Immunohistochemical analysis of SUOX expression is shown in Fig. 1. Evaluation of immunostained samples by the two pathologists was highly reliable and reproducible, with intraclass correlation scores for two distinct measurements of 0.837 (excellent). To produce objective data, quantitative analysis of SUOX expression was performed with open-source NIH ImageJ software, as described previously [25, 26]. A significant correlation

Table 1 Patient's characteristics

Parameter	
Patients, no	97
Age at diagnosis (year), median (range)	68 (56–77)
PSA level at diagnosis (ng/ml)	
Total, median (range)	8.00 (2.13–52.65)
< 10	69 (71.1%)
> 10	28 (28.9%)
D'Amico risk stratification, no (%)	
Low	18 (18.6%)
Intermediate	40 (41.2%)
High	39 (40.2%)
Gleason score, no (%)	
$6 \geq$	15 (15.5%)
3 + 4 = 7	35 (36.1%)
4 + 3 = 7	33 (34.0%)
$8 \leq$	14 (14.4%)
Pathological T stage, no (%)	
T2	69 (71.1%)
T3a	21 (21.7%)
T3b	7 (7.2%)
Lymphatic invasion, no (%)	3 (3.1%)
Peripheral nerve invasion, no (%)	48 (49.5%)
Resection margin positive, no (%)	47 (48.5%)
Ki-67 positive index (%), no (%)	
1 >	70 (72.2%)
1–5	19 (19.6%)
5 <	8 (8.2%)
Follow-up time (month), median (quartile)	60 (46–86)
Biochemical recurrence, no (%)	53 (54.3%)
Presence	53 (54.3%)
None	44 (45.4%)

no number

was observed between the evaluation using image analysis software and that performed by a pathologist applying the Wilcoxon rank sum test ($p < 0.0001$; date not shown). By constructing a receiver-operating characteristic curve, SUOX expression was categorized as high (score: ≥ 11) or low (score: ≤ 10). Of these 97 patients, 35 (36.1%) were categorized in the high-expression group (Fig. 2a, b), and 62 (63.9%) were categorized in the low-expression group (Fig. 2c, d). The correlations between SUOX expression and clinicopathological characteristics are summarized in Table 2. PSA level at diagnosis ($p = 0.017$), lymphatic invasion ($p = 0.012$), and biochemical recurrence ($p < 0.0001$) were more frequently identified in the high-expression group than in the low-expression group. However, there were no correlations between SUOX expression and Gleason score ($p = 0.782$).

Fig. 1 Score map of SUOX expression in 97 cases of prostatic cancer

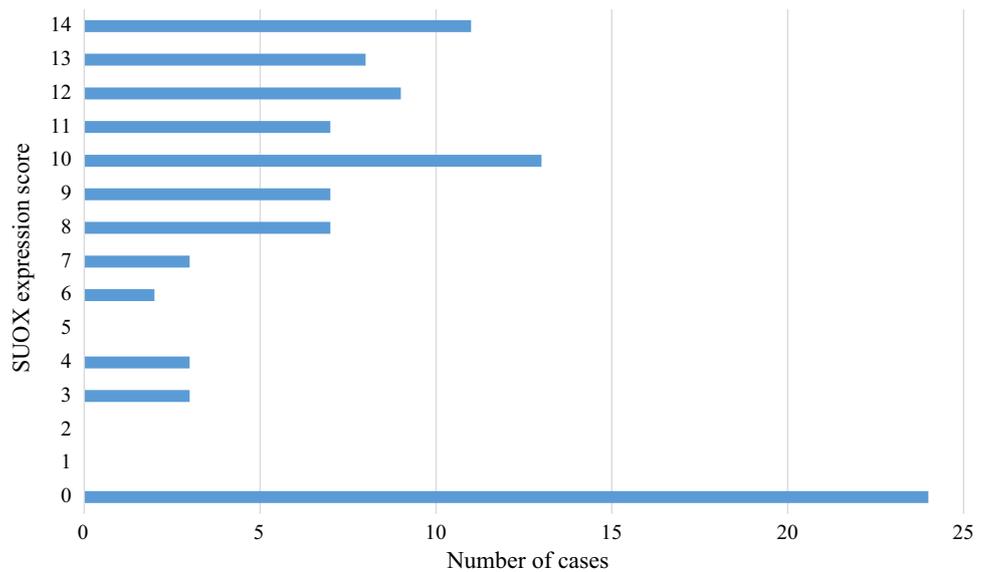
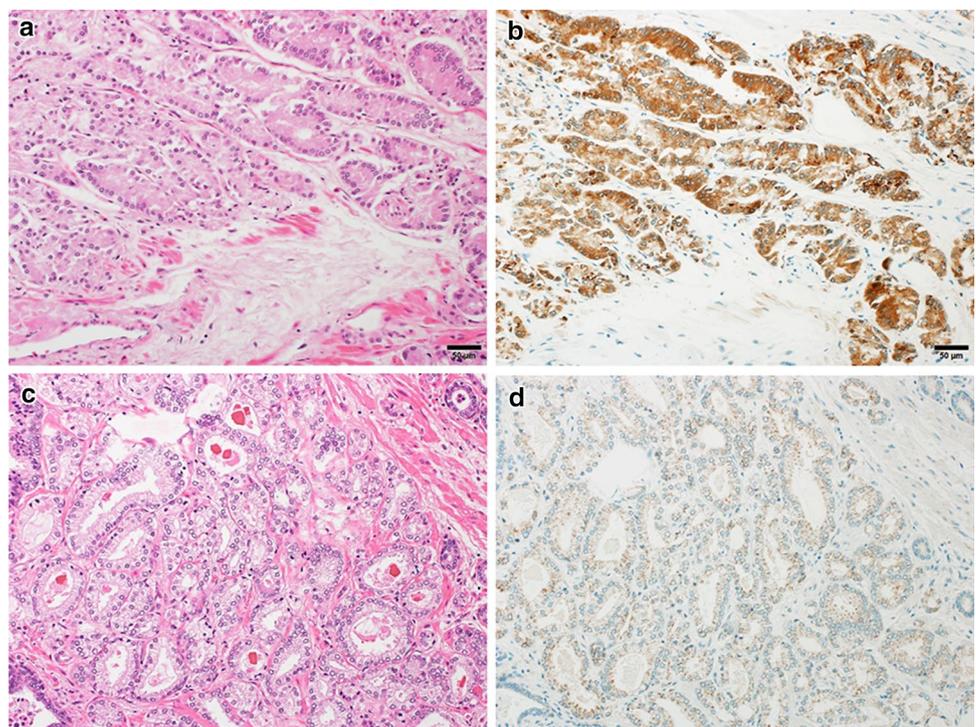


Fig. 2 SUOX expression of prostatic cancer. Photomicrographs of prostatic carcinoma cells stained with hematoxylin–eosin (a, c), or immunostained with SUOX (b, d). SUOX expressions are observed in the cytoplasm. Representative cases of SUOX high- and low-expression groups are shown in b and d, respectively



Identification of SUOX as a biomarker of prostatic biochemical recurrence

Kaplan–Meier curves demonstrated that the time to biochemical recurrence was significantly shorter in patients with high SUOX expression than in those with low SUOX expression ($p = 0.0008$; Fig. 3). Univariate and multivariate analyses of SUOX expression are shown in Table 3. Univariate analysis for time to biochemical recurrence revealed that SUOX expression (high versus low: hazard

ratio [HR]=2.31, 95% confidence interval [CI]=1.35–4.04, $p = 0.0023$), Gleason score (3 + 4 = 7: HR = 2.93, 95% CI = 1.00–12.4; 4 + 3 = 7: HR = 3.33, 95% CI = 1.13–14.2; ≥ 8 : HR = 7.02, 95% CI = 2.15–31.4, $p = 0.0103$), pathological T stage (T3a: HR = 1.90, 95% CI = 1.01–3.44; T3b: HR = 3.44, 95% CI = 1.27–7.84, $p = 0.015$), and resection margin positive (HR = 2.60, 95% CI = 1.49–4.67, $p = 0.0007$) were significant predictors for biochemical recurrence. Moreover, multivariate analysis demonstrated that there were no relationships between biochemical recurrence

Table 2 Correlation between SUOX expression and clinicopathological characteristics

Parameter	Low expression of SUOX, no (%)	High expression of SUOX, no (%)	<i>p</i> value
Patients, no	62 (63.9%)	35 (36.1%)	
Age at diagnosis (year), median (range)	68 (55–75)	66 (53–77)	0.079
PSA level at diagnosis (ng/ml)			
Total, median (range)	6.25 (2.13–62.34)	6.91 (4.29–45.29)	0.017
< 10	46 (74.2%)	23 (65.7%)	0.379
> 10	16 (25.8%)	12 (34.3%)	–
Gleason score			0.782
6 \geq	11 (17.7%)	4 (11.4%)	–
3+4=7	23 (37.1%)	12 (34.3%)	–
4+3=7	20 (32.2%)	13 (37.1%)	–
8 \leq	8 (12.9%)	6 (17.1%)	–
Pathological T stage			0.18
T2	48 (77.4%)	21 (60.0%)	–
T3a	10 (16.1%)	11 (31.4%)	–
T3b	4 (6.5%)	3 (8.6%)	–
Presence of lymphatic invasion	0 (0.0%)	3 (8.6%)	0.012
Presence of peripheral nerve invasion	28 (45.2%)	20 (57.1%)	0.256
Resection margin positive	29 (46.8%)	18 (51.4%)	0.659
Biochemical recurrence			< 0.0001
Presence	24 (38.7%)	29 (82.9%)	–
None	38 (61.3%)	6 (17.1%)	–

no number

Table 3 Univariate and multivariate analyses for time to biochemical recurrence

Parameter	Univariate		Multivariate	
	HR (95% CI)	<i>p</i> value	HR (95% CI)	<i>p</i> value
Age at diagnosis, > 67 (year)	1.22 (0.70–2.15)	0.476		
PSA level at diagnosis, > 10 (ng/ml)	1.72 (0.97–2.97)	0.065		
Gleason score				
6 \geq	1	0.0103	1	0.0947
3+4=7	2.93 (1.00–12.4)		2.61 (0.88–11.2)	
4+3=7	3.33 (1.13–14.2)		2.64 (0.85–11.6)	
8 \leq	7.02 (2.15–31.4)		4.78 (1.39–22.1)	
Pathological T stage				
T2	1	0.015	1	0.367
T3a	1.90 (1.01–3.44)		1.05 (0.52–2.20)	
T3b	3.44 (1.27–7.84)		1.97 (0.68–5.08)	
Presence of lymphatic invasion	2.27 (0.55–6.23)	0.222		
Presence of peripheral nerve invasion	1.67 (0.97–2.94)	0.062		
Resection margin positive	2.60 (1.49–4.67)	0.0007	2.02 (1.08–3.97)	0.283
Ki-67-positive index (%)				
1>	1	0.376		
1–5	1.09 (0.57–2.34)			
5<	1.87 (0.71–4.16)			
High expression of SUOX	2.32 (1.35–4.04)	0.0023	2.33 (1.32–4.15)	0.0037

HR hazard ratio, *CI* confidence interval

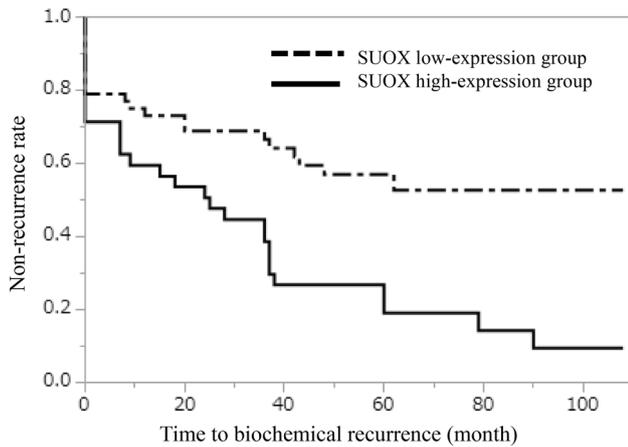


Fig. 3 Relationship between SUOX expression and time to biological recurrence. Time to biochemical recurrence was significantly shorter in patients with high SUOX expression than in those with low SUOX expression ($p=0.0008$)

and Gleason score or pathological T stage. In contrast, SUOX expression level (high versus low: HR = 2.33, 95% CI = 1.32–4.15, $p=0.0037$) and resection margin positive (HR = 2.02, 95% CI = 1.08–3.97, $p=0.0283$) were identified as independent poor prognostic factor for biochemical recurrence.

Correlation between SUOX expression and Ki-67 labeling index in prostate cancer

Next, we investigated the correlation between SUOX expression and Ki-67 LI in prostate cancer. Immunohistochemical

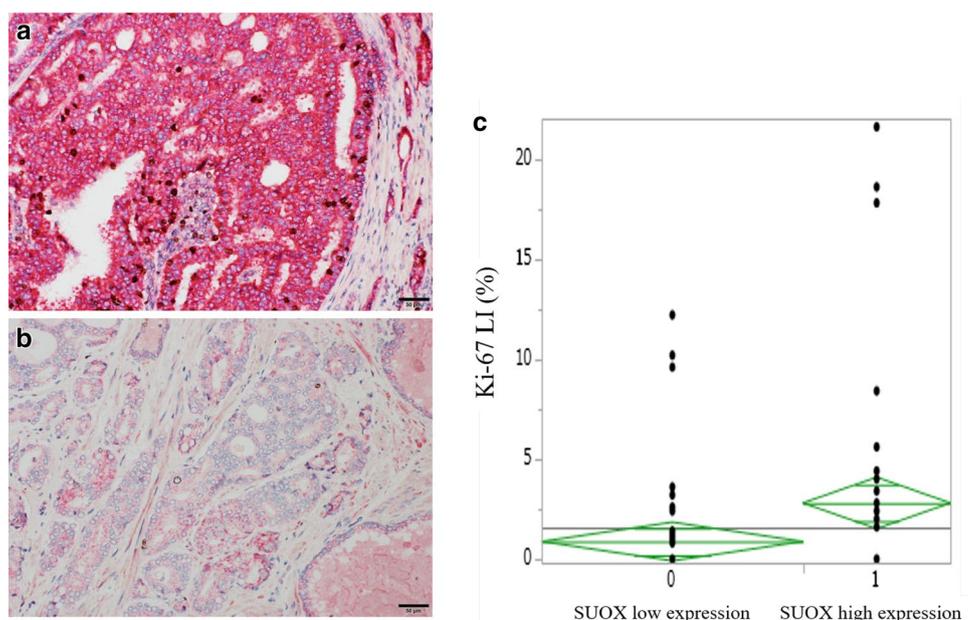
staining revealed high nuclear expression of Ki-67 in tumor cells with strong SUOX expression (Fig. 4a), whereas those with low SUOX expression had only low level of Ki-67 nuclear expression (Fig. 4b). Statistically, the Ki-67 LI was significantly higher in the high SUOX expression group than in the low SUOX expression group ($p=0.0058$; Fig. 4c).

Discussion

In this study, we evaluated SUOX staining in resected prostate cancer tissues, and the feasibility of using the results as a predictive factor with respect to biochemical recurrence was considered. The previous reports have considered PSA level at diagnosis, Gleason score and surgical margin status of resected tumor, pathological T stage, and preoperative PSA as important prognostic factors following radical prostatectomy [27–29]; however, in this study, patients in the SUOX high-expression group exhibited a poor prognosis compared with those in the low-expression group, and the results of univariate and multivariate analyses also suggested that high SUOX expression may be a useful prognostic factor for biochemical recurrence. The Ki-67 LI in the SUOX high-expression group was significantly higher than that in the low-expression group, and the results indicated that SUOX was related to cell proliferative ability. Based on these findings, histological evaluation of SUOX has been shown to be useful for decision-making regarding the postoperative treatment strategy.

Prostate cancer exhibits high heterogeneity, and the proliferation and differentiation potential of prostate cancer cells have been attributed to the involvement of multiple signal

Fig. 4 Comparison of SUOX expression and Ki-67 labeling index in prostate cancer. Immunohistochemical staining revealed high nuclear expression of Ki-67 in tumor cells with strong SUOX expression, (a) whereas those with low SUOX expression had only low level of Ki-67 nuclear expression (b). Statistically, the Ki-67 LI was significantly higher in the high SUOX expression group than in the low SUOX expression group ($p=0.0058$; c)



transduction and metabolic pathways [30–32]. Although the presence of molecules associated with glucose metabolism has been reported to be correlated with prognosis related to abnormal functions and expression in many malignant tumors [33–35], there have been a few reports discussing SUOX as a factor involved in mitochondrial glucose metabolism. SUOX expression in hepatocellular carcinoma decreased in a stepwise manner along with the carcinogenic process, and the expression of SUOX decreased as tumor size increased [22]. In addition, Nakamura et al. found SUOX expression to be diminished in tongue cancer cells compared with healthy tissues, and the expression level continued to decrease as cancerous infiltration progressed [23]. However, a report by Fukushima et al. stated that increases in *SUOX* mRNA in the pancreas were extracted as a poor prognostic factor and that the expression pattern differed depending on the cancer type [36]. In many cancers, hyperglycemia due to the Warburg effect is believed to promote ATP production [37–39]. Recent reports, however, have demonstrated that, when cancer cells proliferate in an aerobic metabolic pathway-dependent manner proximal to the tumor vasculature, lactic acid uptake increases in conjunction with activation of the glycolytic system, and cell proliferation is thought to be accelerated as a result of conversion of lactic acid to pyruvate via the activity of lactate dehydrogenase B and subsequent use of pyruvate as a substrate for oxidative phosphorylation [40]. The Ki-67 LI is recognized as a marker of cell proliferation, and Ki-67 LI scores were significantly higher in the SUOX high-expression group than in the low-expression group in this study, suggesting a relationship with cell proliferative capacity. These findings indicated that cell proliferation may also be induced in prostate cancer as a result of activation of oxidative phosphorylation.

In the present study, SUOX was found to be a prognostic factor, although serum PSA values, Gleason scores, and pathological *T* factor were not. In general, Gleason score is an important prognostic factor used in routine clinical practice, and prognostic stratification based on the Gleason grade classification system proposed in recent years has been more accurate than classification based on conventional Gleason scores [41, 42]. However, in this study, no significant correlation was observed between SUOX expression and Gleason score. Gleason score is a metric used in the histological evaluation of tumor cells. However, it is difficult to characterize the biological properties of tumor cells based on tissue morphology alone. Recent studies have reported that oxidative phosphorylation in the mitochondria of cancer cells is equivalent or greater than that in normal cells [43, 44]. This is consistent with the early biochemical recurrence as well as high Ki-67 LI scores observed in the SUOX high-expression group in this study. In these patients, SUOX expression was found to be a promising biomarker for evaluating malignancies that cannot be identified based on tissue morphology

alone. Overall, our findings indicate the feasibility of using SUOX expression as a biochemical recurrence factor along with Gleason score.

Although local recurrence and distant metastasis may occur after biochemical recurrence, as a clinicopathological factor, positive resection margin is considered the most important predictor of biochemical recurrence [13–15, 45–47]. In fact, in our study, positive resection margin was an independently strong predictor of biochemical recurrence. In addition, high SUOX expression was also demonstrated to serve as a predictor of biochemical recurrence, as reliably as positive surgical margin. In general, biochemical recurrence serves as an indicator of the early stages of relapse [46, 47]; the fact that a large number of tumor cells strongly expressing SUOX correlate with tumor proliferation ability can be hypothesized to suggest that these cells exhibit tumor characteristics that are likely to result in recurrence. Since SUOX expression in resected specimens is as strong as a predictor of biochemical recurrence as positive surgical margin is, the extent of SUOX expression could be useful information in determining postoperative treatment strategy. However, SUOX expression may be difficult to assess in small ranges of tissue owing to heterogeneity, as shown in Table 1. As such, in the future, we will measure SUOX expression in prostate biopsy tissues and add this assessment to the initial diagnostic tests to explore the clinical applications of this marker.

Conclusion

Our findings in this study suggested the superior utility of SUOX immunostaining as a predictor of biochemical recurrence over known prognostic factors in radical prostatectomy cases and highlighted the usefulness of SUOX expression, along with Gleason score and serum PSA value, as a powerful prognostic biomarker for decision-making during postoperative follow-up after total prostatectomy period and for the need for salvage therapy.

Compliance with ethical standards

Conflict of interest All authors have declared that they have no conflicts of interest.

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