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Original contribution

SUOX and GLUT1 are biomarkers for the prognosis in large duct type intrahepatic cholangiocarcinoma☆

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Summary Intrahepatic cholangiocarcinoma (iCCA) is the second most common hepatic malignant disease and has a poor prognosis, but few biomarkers have been found. SUOX is an important factor in energy metabolism and a poor prognostic factor in other malignancies. In this study, we aimed to clarify the relationship between SUOX and GLUT expression in large duct type iCCA and the mechanism of mitochondrial energy metabolism in iCCA. We evaluated SUOX and GLUT1 expression in 96 large duct type iCCA cases and proportion score (PS) was used to evaluate the expression; receiver operating characteristic (ROC) curves of both SUOX and GLUT1 expression were generated, and the Kaplan–Meier method and Cox regression analysis were used to estimate overall survival. Of the 96 iCCA cases, 73 (76.0%) showed low SUOX expression and 66 (68.8%) showed high GLUT1 expression. The 5-year survival rate of iCCA with low SUOX expression was significantly shorter than that of

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iCCA with high SUOX expression (p = 0.001). In contrast, the 5-year survival rate of iCCA with high GLUT1 expression was significantly shorter than that of iCCA with low GLUT1 expression (p = 0.005). According to Spearman's correlation, there was no correlation between SUOX and GLUT1. Conversely, the combination of low SUOX and high GLUT1 expression was the most common in 51 of 96 cases (53.1%), and the overall survival was significantly shorter than that of patients with other combinations. Furthermore, SUOX was shown to be an independent prognostic factor together with GLUT1, suggesting that SUOX in combination with GLUT1 can predict the prognosis of large duct type iCCA.

1. Introduction

Intrahepatic cholangiocarcinoma (iCCA) is the second most common primary liver cancer after hepatocellular carcinoma, and its incidence is increasing in many countries, including Japan [1−5]. In Japan, iCCA accounts for 4.77% of primary liver cancer [6]. The 5-year survival rate of hepatocellular carcinoma is 50.4%, which is a dramatic improvement compared to 40 years ago. In contrast, iCCA is a malignant disease with a poor prognosis, with a 5-year survival rate of only 30.9%, although it has been gradually improving due to advances in early diagnosis and treatment [6]. Guglielmi et al. reported that a mixed gross classification of mass-forming and peribiliary invasion, lymph node metastasis, and vascular invasion are poor prognostic factors of iCCA [7]. Although there are many clinicopathological studies on iCCA, there are few studies on biomarker characterization. Therefore, we investigated potential prognostic biomarkers to characterize the biology of iCCA.

Glucose transporter 1 (GLUT1), one of the biomarkers of iCCA, is an important factor in energy metabolism, especially glucose metabolism, and is known to influence prognosis [8]. Glucose is imported into the cytoplasm of the cell via glucose transporters, such as GLUT1. GLUT1 expression is known to be upregulated in many cancers, including breast, esophageal, prostate, oral squamous cell, endometrial, thyroid, gastric, colorectal cancer, and non-small cell lung cancer, as well as bile duct cancer [9−18]. The mitochondrial energy metabolism is different in cancer cells compared to that in normal cells. Glucose is metabolized to pyruvate in the cytoplasm via glycolysis. Under aerobic conditions, pyruvate is taken up by mitochondria, where aerobic energy production occurs. However, in cancer cells, even under aerobic conditions, oxygen-based oxidative phosphorylation in mitochondria is suppressed and energy production by the glycolytic system in the cytosol is enhanced. This is called the Warburg effect and is observed in many carcinomas [19,20]. Thus, mitochondria play an important role in energy production, and the metalloenzyme sulfite oxidase (SUOX) is believed to play an important role in mitochondria-centered ATP production.

SUOX is an enzyme present in mitochondria that converts sulfite to sulfate. The electrons produced in this process are transferred to the electron transport system via cytochrome *c* and used for ATP production by oxidative phosphorylation. SUOX has been reported to be an important prognostic factor in hepatocellular carcinoma, oral squamous cell carcinoma, prostate cancer, and gastric cancer [21−24]. However, there is still no report on the role of SUOX expression in iCCA. Therefore, in this study, we aimed to elucidate the relationship between SUOX and GLUT1 and the mechanism of energy metabolism in mitochondria in iCCA.

2. Materials and methods

2.1. Patients and tissue samples

According to the current World Health Organization (WHO) classification, iCCA has two main subtypes: large duct and small duct [25]. Large duct type iCCA arises in the large intrahepatic bile ducts near the hepatic hilus and resembles perihilar and extrahepatic cholangiocarcinoma [25]. We enrolled 96 consecutive patients, diagnosed with large duct type iCCA, after surgery at Kurume University Hospital between 2000 and 2018, and a clinicopathological study was conducted. This study received the approval of the Research Ethics Committee of the Kurume University (#18068) and Saga Prefectural Hospital Koseikan, and conforms to the guidelines of the Declaration of Helsinki. An opt-out policy was used in this study. Slides of surgical samples obtained from patients with iCCA referred to Kurume University Hospital were eligible.

We cut all sections at a thickness of 4 μm and stained them with hematoxylin and eosin. In this study, we further classified large duct type iCCA into two subtypes: large duct type iCCA with no or minimal perihilar invasion (NMPI) type and that with extensive perihilar invasion (EPI) type. Pathological factors evaluated were histology (Fig. 1a−c), vascular invasion, soft part invasion, perineural invasion, pStage, and Ki-67 positivity rate. For perineural invasion, we defined "perineural invasion of the peri-bile duct wall" as perineural invasion around the bile duct

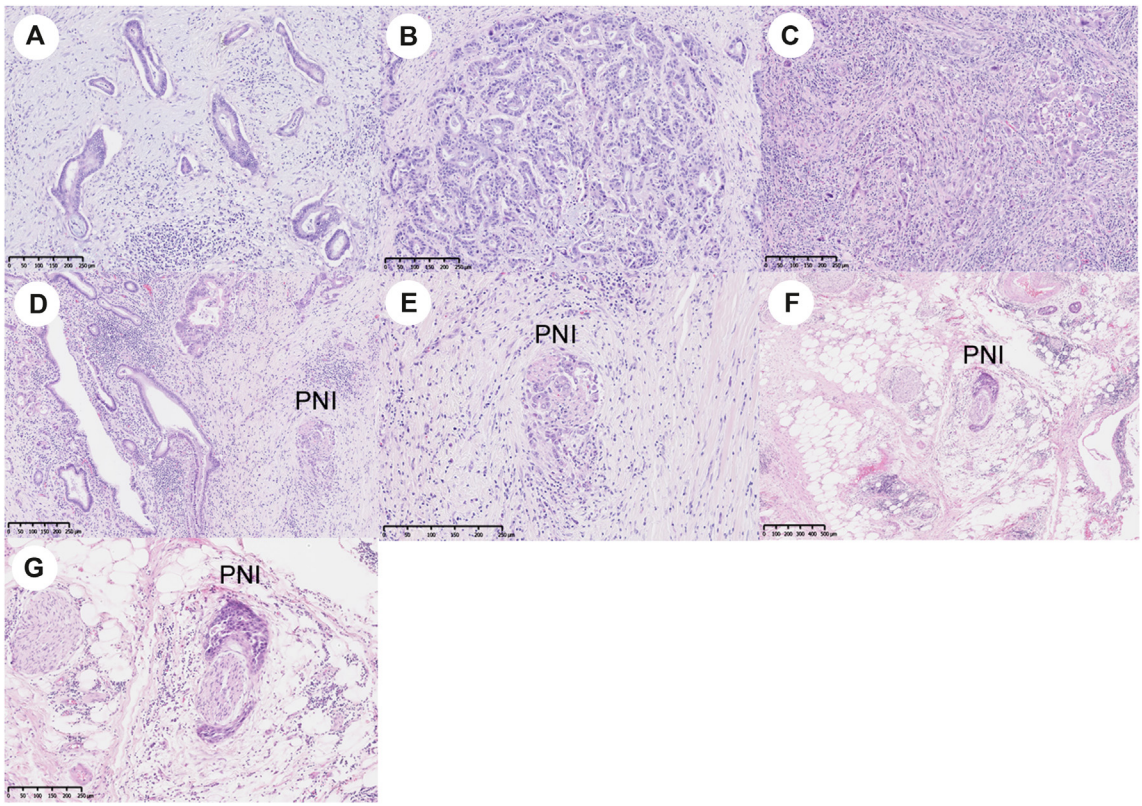

Fig. 1 Microscopic findings of large duct type iCCA (a: well-differentiated, b: moderately differentiated, c: poorly differentiated) stained with hematoxylin-eosin and perineural invasion (d, e: peri-bile duct, f, g: outside). iCCA: intrahepatic cholangiocarcinoma; PNI: perineural invasion.

wall (Fig. 1d and e) and "perineural invasion outside the bile duct wall" as perineural invasion in the fat tissue, away from the bile duct wall (Fig. 1f and g). Perineural invasion was evaluated in 94 cases because of the difficulty in evaluating 2 cases due to poor specimens. pStage classification was performed according to the 8th edition of the Union for International Cancer Control (UICC) tumor, nodes, and metastases (TNM) Classification.

2.2. Immunohistochemical analysis

In this study, we performed immunohistochemistry (IHC) analysis on paraffin-embedded sections. Paraffin-embedded surgical sections were cut to a thickness of 4 μm, mounted on coated glass slides, and labeled with anti-SUOX (× 600, Abcam, Cambridge, MA, USA), anti-GLUT1 (dilution 1:100, NEO MARKER) and anti-Ki-67 (NCL-Ki67-MM1, dilution 1:200, Leica Biosystems, Nussloch, Germany) antibodies using BenchMark ULTRA (Ventana Automated Systems, Inc., Tucson, AZ, USA). Briefly, for SUOX, GLUT1 and Ki-67, slides were heat-treated using Ventana's ULTRA cell conditioning 1 retrieval solution (CC1, Ventana Automated Systems, Inc., Tucson, AZ, USA) for 36 and 64 min at 95 °C (SUOX and Ki-67), for 20 min and 36 min at 95 °C (GLUT1), and incubated with antibodies anti-SUOX for 32 min, anti-GLUT1 for 32 min, and anti-Ki-67 for 32 min at 37 °C. The automated system used the Ventana UltraVIEW DAB detection kit with the horseradish peroxidase (HRP) enzyme directly conjugated to the secondary antibody and 3,3'-diaminobenzidine (DAB) as the chromogen. Each slide was incubated with the corresponding secondary antibody for 30 min at 37 °C.

We only evaluated the cytoplasmic expression of SUOX and the cytoplasmic membrane expression of GLUT1. SUOX usually stains the cytoplasm of hepatocytes and bile duct epithelial cells. GLUT1 does not stain hepatocytes or bile duct epithelial cells but stains the red blood cells in the background (Fig. 2a and b). The expression of SUOX and GLUT1 in tumor cells was evaluated by population score (PS) depending on their respective staining ratio (PS0: 0%, PS1: 0−1%, PS2: 1−10%, PS3: 10−33%, PS4: 33−66%, PS5: 66−100%). The intensity was not considered. To obtain the cut-off value for SUOX and GLUT1, time-dependent receiver operating characteristic (ROC) curve analysis was performed. By constructing a ROC curve, SUOX and GLUT1 expression was graded as follows: low-expression, $\leq$ PS3 staining of the neoplastic cells or cells with high-expression, $\geq$ PS4 staining of the neoplastic cells. Ki-67 labeling index was calculated as the percentage

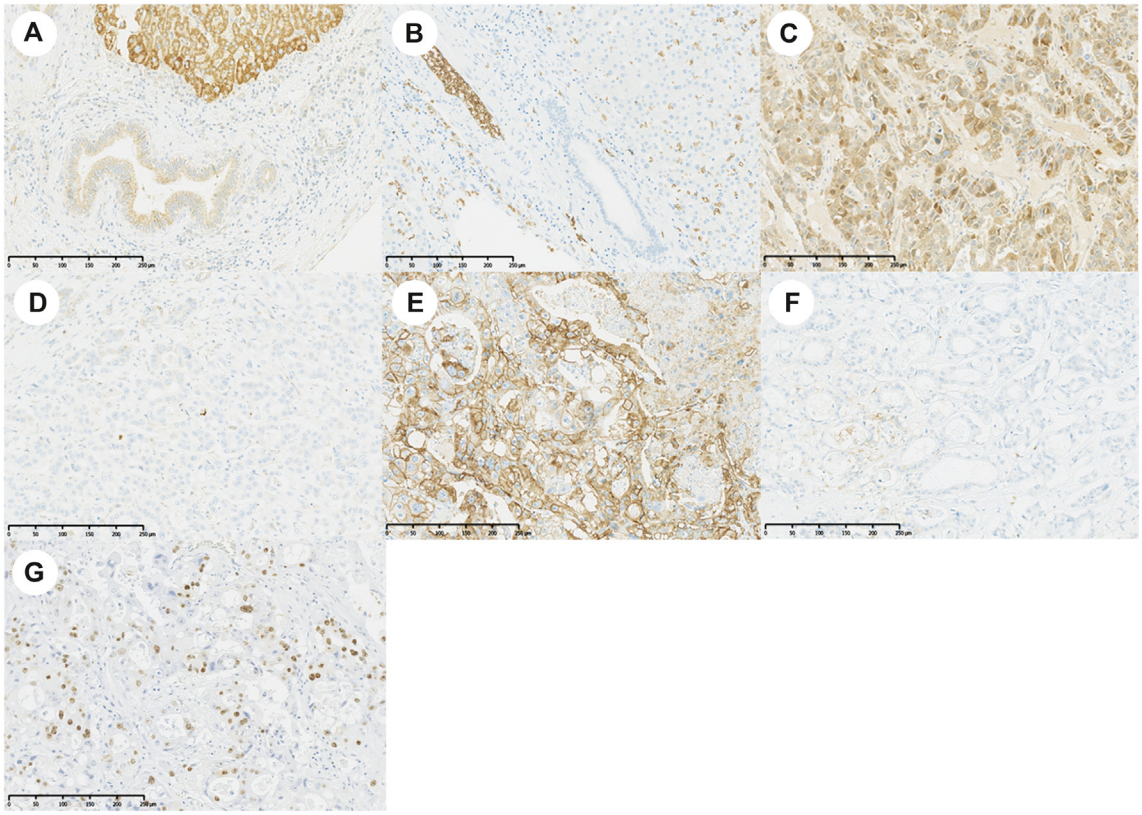

Fig. 2 Microscopic findings of non-neoplastic bile duct and hepatocyte with SUOX (a) and immunostained GLUT1 (b). SUOX expression was observed in the cytoplasm of bile duct. GLUT1 expression was observed in the membrane of blood cells, but was not observed in the cytoplasm of non-neoplastic bile duct and hepatocytes. High and low SUOX expression groups are shown in (c) and (d), respectively. High and low expression GLUT1 groups were shown in (e) and (f), respectively. The percentage of Ki-67-positive tumor nuclei (Ki-67 index) was evaluated (g).

of tumor cells that showed positive expression in the area with the highest Ki-67 expression level (hot spot). They were reviewed by two pathologists (Y.K and Y.N).

2.3. Statistical analysis

Data are presented as medians (interquartile ranges: IQR) or as numbers (percentages). The Cox regression model was applied to evaluate the effects of clinicopathological factors while adjusting for potential confounding factors. As possible explanatory variables, all clinicopathological factors with p values < 0.10 in the univariate analyses and degree of perihilar invasion were included in the model. Comparisons between groups were made using the Mann−Whitney U test for continuous variables and the χ^2 test or Fisher's exact probability test for categorical data. Correlation between SUOX and GLUT1 were examined by the Spearman rank correlation test. The overall survival (OS) was defined as the period between the date of surgery until the date of death due to any cause. OS was estimated using the Kaplan−Meier method. The log-rank test was used to assess differences between groups, and the Bonferroni method was applied for multiple pairwise comparisons. The Cox regression model was applied to evaluate the effects of clinicopathological factors while adjusting for potential confounding factors. Data were analyzed using R version 4.1.2 and statistical significance was set at p value < 0.05.

3. Results

3.1. Patient characteristics

The clinicopathologic findings of the 96 cases retrieved from the patients' medical records are shown in Table 1. The histological types were well differentiated in 44 cases (45.8%), moderately differentiated in 46 cases (47.9%), and poorly differentiated in 6 cases (6.2%). Vascular invasion was observed in 65 cases (67.7%) and soft part invasion was observed in 63 cases (65.6%). In addition, 30 cases (31.9%) showed perineural invasion of the peri-bile duct wall and 45 cases (47.9%) showed perineural invasion outside the bile duct wall; 25 cases (26.0%) were positive for margins. pStage classification was pStage IA in 24 cases (25.0%), pStage II in 45 cases (46.8%), and pStage IIIB in 27 cases (28.1%). The median follow-up period was 544.50 days, and the number of deaths during the follow-up period was 47 (49.0%).

Table 1 Clinicopathological findings of patients with large duct type iCCA.

	n	%
Patients	96	
Age: <65/≥65	31/65	32.3/67.7
Gender: Male/Female	64/32	66.7/33.3
NMPI subtype/EPI subtype	46/50	47.9/52.1
Tumor size [cm], median (IQR)	2.6 (2.0, 4.0)	
Histology: G1/G2/G3	44/46/6	45.8/47.9/6.2
Vascular invasion	65	67.7
Soft part invasion	63	65.6
PNI: Peri-bile duct /Outside	30/45	31.9/47.9
Surgical margin, positive	25	26.0
pStage: IA/II/IIIB	24/45/27	25.0/46.9/28.1
Background liver (CHB /CHC/PI/UK)	13/10/1/72	13.5/10.4/1.0/75.0
Ki-67 labeling index (median [IQR])	16.25 [6.68, 27.50]	
Overall survival [day], median (IQR)	544.5 (319.00, 111.25)	
Survival: Alive/Dead	49/47	51.0/49.0

Abbreviations: iCCA, intrahepatic cholangiocarcinoma; NMPI, large duct type iCCA with no or minimal perihilar invasion; EPI, extensive perihilar invasion; G1, well-differentiated; G2, moderately differentiated; G3, poorly differentiated; PNI, perineural invasion; IQR, interquartile range; CHB, chronic hepatitis B infection; CHC, chronic hepatitis C infection; NBNC, PI, parasite infection; and UK, unknown.

3.2. Immunohistochemical analysis of SUOX and GLUT1 expression

SUOX expression was high in 23 of 96 patients (24.0%) (Fig. 2c) and low in 73 of 96 patients (76.0%) (Fig. 2d). The association between SUOX expression and various clinicopathological factors is shown in Table 2; only perineural invasion was found to be associated with SUOX expression ($p = 0.014$). In SUOX low expression group, perineural invasion outside the bile duct wall was more common. In comparison, GLUT1 was frequently highly expressed, with 66 of 96 cases (68.7%) showing a high expression (Figs. 2e) and 30 of 96 cases (31.3%) showing a low expression (Fig. 2f). The association between GLUT1 expression and various clinicopathological factors is shown in Table 3. GLUT1 was associated with degree of perihilar invasion ($p = 0.016$), soft part invasion ($p = 0.038$), perineural invasion ($p = 0.044$), and Ki-67 positivity rate ($p = 0.038$) (Fig. 2g). Perineural invasion outside the bile duct wall was more common in high GLUT1 expression group. Histology, vascular invasion, and margin were not associated with either SUOX or GLUT1 expression.

3.3. Univariate and multivariate analyses of the OS

The results of the Cox regression analysis for OS are shown in Table 5. In univariate analysis, soft part invasion (yes vs. no; hazard ratio (HR) = 3.478, 95% confidence interval (CI) 1.704, 7.098, $p = 0.001$), perineural invasion (outside vs. peri-bile duct or no; HR = 2.827, 95% CI 1.549, 5.161, $p = 0.001$), pStage (pStage IIIB vs. pStage II/IA; HR = 1.825 95% CI 1.007, 3.309, $p = 0.048$), SUOX expression (low expression vs. high expression; HR = 3.887 95% CI 1.664, 9.188, $p = 0.002$), and GLUT1 Expression (high expression vs. low expression; HR = 2.638 95% CI 1.304, 5.335 $p = 0.007$) were extracted as prognostic factors. In the Kaplan−Meier curve the 5-year survival rates of patients with low and high SUOX-expressing iCCA were 21.1% and 72.7%, respectively, with those of patients with low SUOX-expressing iCCA being significantly shorter ($p = 0.001$) (Fig. 3a). The 5-year survival rates of patients with high and low GLUT1-expressing iCCA were 21.2% and

Table 2 Correlation between SUOX expression and clinicopathological characteristics.

	SUOX (L)		SUOX (H)		p-value
	N	%	n	%	
Patients	73		23		
Age: <65/≥65	22/51	30.1/69.9	9/14	39.1/60.9	0.451
Gender: Male/Female	50/23	68.5/31.5	14/9	60.9/39.1	0.613
NMPI subtype/EPI subtype	34/39	46.6/53.4	12/11	52.2/47.8	0.811
Tumor size [cm]: <2.5/≥2.5	29/44	39.7/60.3	12/11	52.2/47.8	0.339
Histology: G1/G2, G3	32/41	43.8/56.2	12/11	52.2/47.8	0.632
Vascular invasion	50	68.5	15	65.2	0.801
Soft part invasion	51	69.9	12	52.2	0.137
PNI: None, Peri-bile duct/Outside	32/39	45.1/54.9	17/6	73.9/26.1	0.014
Surgical margin, positive	19	26.0	6	26.1	1.000
pStage: IA,II/IIIB	51/22	69.9/30.1	18/5	78.3/21.7	0.596
Ki-67 labeling index (median [IQR])	16.20 [5.60, 26.40]		16.80 [13.00, 31.90]		0.14
GLUT1: L/H	22/51	30.1/69.9	8/15	34.8/65.2	0.797

Abbreviations: NMPI, large duct type iCCA with no or minimal perihilar invasion; EPI, extensive perihilar invasion; G1, well-differentiated; G2, moderately differentiated; G3, poorly differentiated; PNI, perineural invasion; L, low expression; H, high expression; and IQR, interquartile range.

Table 3 Correlation between GLUT1 expression and clinicopathological characteristics.

	GLUT1(L)		GLUT1(H)		
	N	%	n	%	
Patient	30		66		
Age: <65/≥65	13/17	43.3/56.7	18/48	27.3/72.7	0.158
Gender: Male/Female	19/11	63.3/36.7	45/21	68.2/31.8	0.648
NMPI subtype/EPI subtype	20/10	66.7/33.3	26/40	39.4/60.6	0.016
Tumor size [cm]: <2.5/≥2.5	14/16	46.7/53.3	27/39	40.9/59.1	0.659
Histology: G1/G2, G3	10/20	33.3/66.7	14/52	21.2/78.8	0.190
Vascular invasion	17	56.7	48	72.7	0.158
Soft part invasion	15	50	48	72.7	0.038
PNI: None, Peri-bile duct/Outside	20/9	69.0/31.0	29/36	44.6/55.4	0.044
Surgical margin, positive	4	13.3	21	32.3	0.079
pStage: IA, II/IIIB	25/5	83.3/16.7	44/22	66.7/33.3	0.141
Ki-67 labeling index (median [IQR])	9.10 [4.40, 20.40]		17.65 [9.72, 29.85]		0.038
SUOX: L/H	22/8	73.3/26.7	51/15	77.3/22.7	0.797

Abbreviations: NMPI, large duct type iCCA with no or minimal perihilar invasion; EPI, extensive perihilar invasion; G1, well-differentiated; G2, moderately differentiated; G3, poorly differentiated; PNI, perineural invasion; L, low expression; H, high expression; and IQR, interquartile range.

61.6%, respectively, with those of patients with high GLUT1-expressing iCCA being significantly shorter ($p = 0.005$) (Fig. 3b). The multivariate analysis also showed that SUOX expression (low expression vs. high expression; HR = 3.287 95% CI 1.366, 7.907, $p = 0.008$) and GLUT1 expression (high expression vs. low expression; HR = 2.504 95% CI 1.066, 5.884 $p = 0.035$) were the only independent prognostic factors.

3.4. Relationship between OS, SUOX expression, and GLUT1 expression

Spearman's correlation analysis showed no correlation between SUOX and GLUT1 expression (R = a-0.026). In contrast, the combination of low expression of SUOX and high expression of GLUT1 was the most common in 51 of 96 cases (53.1%) (Table 4), with significantly shorter OS than the other combinations (log-rank test with Bonferroni correction: low SUOX/low GLUT1: $p = 0.024$ vs. high SUOX/high GLUT1: $p = 0.003$ vs. high SUOX/low GLUT1: $p = 0.009$) (Fig. 3c).

4. Discussion

We examined the expression of SUOX and GLUT1 immunohistochemically in 96 cases of large duct type iCCA. The incidence of low SUOX expression was 76.0%, indicating that SUOX-mediated altered mitochondrial function occurs in many iCCA cases. In contrast, GLUT1, which

Table 4 Correlation between combination of GLUT1 and SUOX expression and clinicopathological characteristics.

	GLUT1(L)/SUOX(L)	GLUT1(L)/SUOX(H)	GLUT1(H)/SUOX(L)	GLUT1(H)/SUOX(H)	p -value
Patient (n)	22	8	51	15	
Age: <65/≥65 (%)	3/6 (33.3/66.7)	7/11 (38.9/61.1)	7/23 (23.3/76.7)	14/25 (35.9/64.1)	0.614
Gender: Female/Male (%)	15/7 (68.2/31.8)	4/4 (50.0/50.0)	35/16 (68.6/31.4)	10/5 (66.7/33.3)	0.766
NMPI subtype/EPI subtype (%)	14/8 (63.6/36.4)	6/2 (75.0/25.0)	20/31 (39.2/60.8)	6/9 (40.0/60.0)	0.093
size (cm, median [IQR])	2.65 [2.05, 4.02]	2.70 [2.00, 3.78]	2.60 [2.00, 4.00]	2.30 [1.95, 3.25]	0.868
Histology: G1/G2/G3 (%)	11/9/2 (50.0/40.9/9.1)	6/2/0 (75.0/25.0/0.0)	21/26/4 (41.2/51.0/7.8)	6/9/0 (40.0/60.0/0.0)	0.572
Soft part invasion: −/+ (%)	10/12 (45.5/54.5)	5/3 (62.5/37.5)	12/39 (23.5/76.5)	6/9 (40.0/60.0)	0.069
Vascular invasion: −/+ (%)	9/13 (40.9/59.1)	4/4 (50.0/50.0)	14/37 (27.5/72.5)	4/11 (26.7/73.3)	0.433
PNI: None/Peri-bile duct/Outside (%)	4/9/8 (19.0/42.9/38.1)	5/2/1 (62.5/25.0/12.5)	8/11/31 (16.0/22.0/62.0)	2/8/5 (13.3/53.3/33.3)	0.012
surgical margin: −/+ (%)	18/4 (81.8/18.2)	8/0 (100.0/0.0)	36/15 (70.6/29.4)	9/6 (60.0/40.0)	0.152
pStage: IA/II/IIIB (%)	7/11/4 (31.8/50.0/18.2)	3/4/1 (37.5/50.0/12.5)	11/22/18 (21.6/43.1/35.3)	3/8/4 (20.0/53.3/26.7)	0.692
Ki-67 labeling index (median [IQR])	6.70 [4.30, 11.60]	8.55 [4.02, 16.78]	19.20 [8.00, 28.90]	22.10 [12.75, 30.80]	0.008

Abbreviations: NMPI, large duct type iCCA with no or minimal perihilar invasion; EPI, extensive perihilar invasion; G1, well-differentiated; G2, moderately differentiated; G3, poorly differentiated; PNI, perineural invasion; L, low expression; H, high expression; and IQR, interquartile range.

Table 5 Cox regression analysis for Prognosis of large duct type iCCA.

Parameter	Univariate analysis		Multivariate analysis	
	HR (95% CI)	p-value	HR (95% CI)	p-value
Age (≥65)	1.582 (0.833, 3.005)	0.161		
Gender (Female/Male)	0.665 (0.351, 1.263)	0.212		
NMPI subtype/EPI subtype	1.532 (0.844, 2.781)	0.161	0.736 (0.352, 1.541)	0.417
Size (cm) (≥2.5/<2.5)	0.953 (0.810, 1.120)	0.558		
Histology (G3/G2, G1)	1.038 (0.583, 1.846)	0.900		
Soft part invasion	3.478 (1.704, 7.098)	0.001	2.271 (0.839, 6.146)	0.106
Vascular invasion	1.053 (0.580, 1.912)	0.865		
PNI (Outside/None, Peri-bile duct)	2.827 (1.549, 5.161)	0.001	1.256 (0.515, 3.064)	0.616
pStage (IIIB/IA, II)	1.825 (1.007, 3.309)	0.048	1.26 (0.674, 2.356)	0.469
Surgical margin	1.393 (0.744, 2.609)	0.300		
Ki-67 labeling index (10% increments)	0.815 (0.656,1.014)	0.066	0.809 (0.632,1.036)	0.093
SUOX: L/H	3.887 (1.664, 9.188)	0.002	3.287 (1.366, 7.907)	0.008
GLUT1: H/L	2.638 (1.304, 5.335)	0.007	2.504 (1.066, 5.884)	0.035

Abbreviations: iCCA, intrahepatic cholangiocarcinoma; NMPI, large duct type iCCA with no or minimal perihilar invasion; EPI, extensive perihilar invasion; G1, well-differentiated; G2, moderately differentiated; G3, poorly differentiated; PNI, perineural invasion; L, low expression; H, high expression; HR, hazard ratio; and CI, confidence interval.

reflects sugar uptake, was highly expressed in 68.7% of the patients, suggesting the Warburg effect. SUOX was shown to be an independent prognostic factor in iCCA in both univariate and multivariate analyses, and the case group with low SUOX expression and high GLUT1 expression had a poor prognosis. Additionally, in the multivariate analysis, no other prognostic factors were identified besides SUOX and GLUT1. In previous reports of iCCA, vascular invasion and lymph node metastasis were identified as prognostic factors [7], but in our study they were not found to be independent prognostic factors in multivariate analysis. In a clinicopathological study of iCCA from the viewpoint of location [26], the authors reported that there was no significant difference in median survival between iCCA and perihilar cholangiocarcinoma, and similarly in our study, degree of perihilar invasion of large duct type iCCA was not a prognostic factor. In summary, although many clinicopathologic factors have been identified as prognostic factors for iCCA, our study suggests that mitochondrial function and energy metabolism may also be important prognostic factors.

In the study, many iCCA cases showed low expression of SUOX. In normal cells, aerobic energy production in mitochondria occurs in an aerobic environment; however, in iCCA, aerobic energy production by mitochondria is likely reduced. This was most likely due to the Warburg effect, in which the aerobic energy production in mitochondria is arrested and the glycolytic system metabolism is enhanced in cancer cells, even under aerobic conditions. Low SUOX expression group had a poorer prognosis than high SUOX expression group. SUOX has been reported as an important prognostic factor in various carcinomas, and in gastric and oral squamous cell carcinomas, SUOX shows a tendency for low expression in advanced carcinomas [22,24]. In addition, SUOX has been shown to associate with the degree of differentiation in hepatocellular carcinoma, suggesting that SUOX may be involved in tumor development and progression [21]. The mechanism by which SUOX contributes to cancer progression needs to be elucidated further. In our study, SUOX was an independent prognostic factor, and the results were consistent with previous reports, suggesting that SUOX may be an effective biomarker.

In this study, GLUT1 was also shown to be a prognostic factor; its overexpression may allow excess glucose to enter the pentose-phosphate circuit, producing substances necess ary for fatty acid and nucleic acid syntheses, which in turn activate cell proliferation. In fact, an increase in the pentose-phosphate circuit has been reported in cholangiocarcinoma along with an increase in GLUT1 expression [27], and in our study, the cases with high GLUT1 expression tended to be significantly more positive for Ki-67 than those with low expression. It is expected that by increasing the expression of GLUT1, increased amounts of glucose, necessary for cell proliferation, is taken up by the cells. The results suggest that, as with SUOX, there is an impact on prognosis, but no direct relationship was found between SUOX and GLUT1 expression. On the other hand, the combination of low SUOX expression and high GLUT1 expression had the poorest prognosis. One reason could be that the activation of cell proliferation by high expression of GLUT1 and the Warburg effect by low expression of SUOX may have influenced the prognosis. Although the results of our study only show a partial view of the mitochondria-centered mechanism of energy production, the results indicate that SUOX and GLUT1 are important factors in energy metabolism.

This study has several limitations. First, there are only a few cases of high SUOX expression. Although it is clear that most patients with iCCA have low SUOX expression, and the conducted statistical analysis shows it to be a prognostic factor, we may not be able to fully analyze the clinicopathological characteristics of the high SUOX-expressing group. For this reason, further study of a large number of cases is necessary. Second, it is suggested that the roles of SUOX are

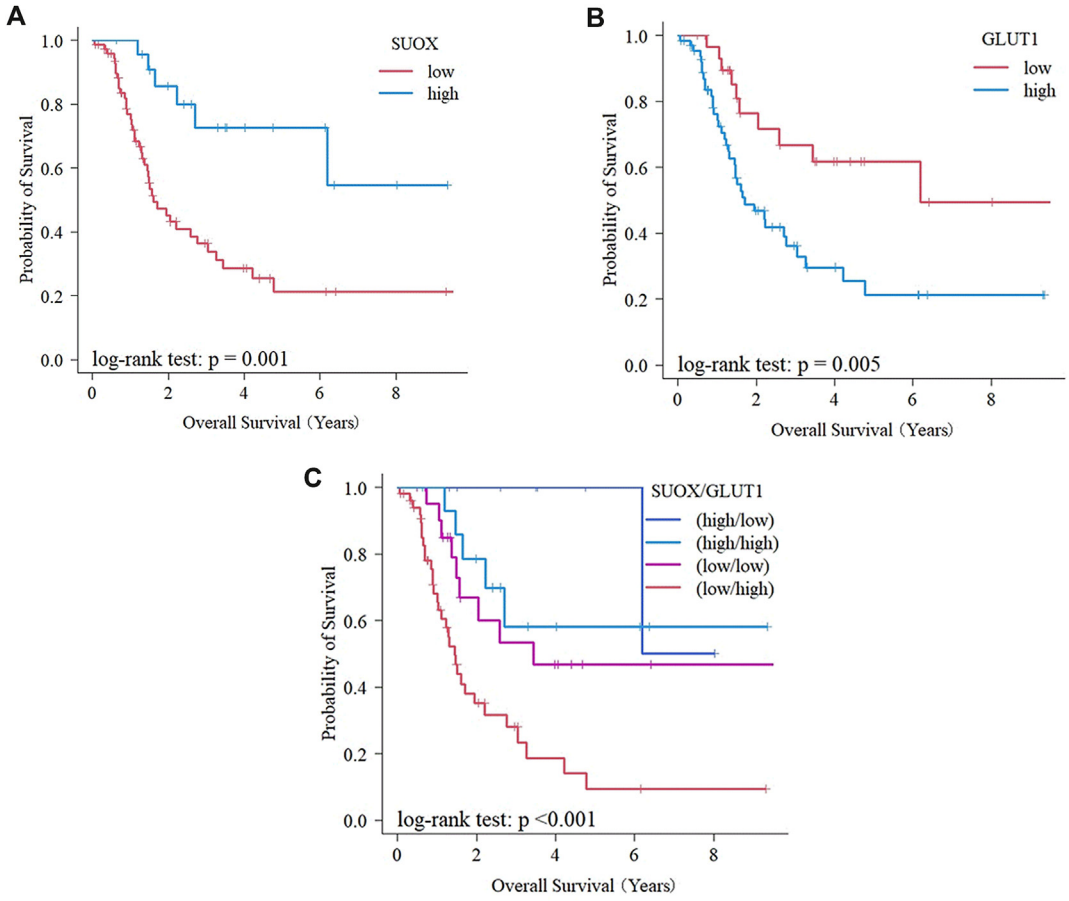

Fig. 3 Kaplan−Meier curves demonstrate that the time to OS is significantly shorter in patients with low SUOX expression than in those with high SUOX expression ($p = 0.001$) (a). Kaplan−Meier curves demonstrate that the time to OS is significantly shorter in patients with high GLUT1 expression than in those with low GLUT1 expression ($p = 0.005$) (b). Kaplan−Meier curves demonstrate that the time to OS is significantly shorter in patients with the combination of low SUOX expression and high GLUT-1 expression than in those with other combinations of the SUOX and GLUT1 expression (low SUOX/low GLUT1: $p = 0.024$ vs. high SUOX/high GLUT1: $p = 0.003$ vs. high SUOX/low GLUT1: $p < 0.001$) (c). OS: overall survival.

different between the organs. In prostate cancer, high SUOX expression was an independent poor prognostic factor [23] which is different from its role in iCCA. In addition, a correlation with tumor growth potential has been reported in prostate cancer [23]; however, we found no relationship between SUOX and Ki-67 expression in iCCA and another study also reported the same in gastric cancer [24]. These results suggest that the relationship between cell proliferative capacity and SUOX expression may vary between the organs. In conclusion, this study showed that SUOX and GLUT1 were independent prognostic factors in iCCA, with no correlation between them. Therefore, molecular pathology studies in various carcinomas should be pursued in the future.

Author contributions

Y.K and Y.N conceptualized this study. Y.K, Y.N and E.S managed the data. E.S was in charge of formal analysis. Y.K, Y.N, J.A, M.N, M.T, T.H and Y.O conducted the study. Y.K, Y.N, and H.Y provided project management. Y.K and Y.N prepared the manuscript. The manuscript was reviewed and revised by H.Y, and the final version was approved for submission by all authors.

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