Histopathology

Histopathology 2016, **68**, 693–701. DOI: 10.1111/his.12806

Spalt-like transcription factor 4 immunopositivity is associated with epithelial cell adhesion molecule expression in combined hepatocellular carcinoma and cholangiocarcinoma

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Date of submission 7 December 2014
Accepted for publication 8 August 2015
Published online *Article Accepted* 12 August 2015

Tanaka Y, Aishima S, Kohashi K, Okumura Y, Wang H, Hida T, Kotoh K, Shirabe K, Maehara Y, Takayanagi R, & Oda Y.
(2016) *Histopathology* **68**, 693–701. DOI: 10.1111/his.12806

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Aim: Combined hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC) (cHCC-CC) is a rare biphasic liver cancer. Recent studies have demonstrated that cHCC-CC originates from hepatic progenitor cells (HPCs). Spalt-like transcription factor 4 (SALL4) is a marker for a progenitor subclass of HCC with an aggressive phenotype. However, little has been revealed about SALL4 expression in cHCC-CC. The aims of this study were to report SALL4 immunopositivity and the results of clinicopathological analysis in cHCC-CC, and to examine the two different nuclear immunostaining patterns for SALL4.

Methods and results: We defined the diffuse finely granular nuclear immunostaining pattern as immunopositive for SALL4; this was observed in eight (8.9%) of 90 cHCC-CCs. SALL4 immunopositivity was significantly associated with immunopositivity for α-fetoprotein, glypican 3, and epithelial cell adhesion molecule (EpCAM). There was no relationship between SALL4 immunopositivity and prognosis. We confirmed SALL4 mRNA expression in samples with a punctuate/clumped immunostaining pattern, which showed a significantly lower rate of immunopositivity for EpCAM than those with a diffuse finely granular pattern.

Conclusions: SALL4 immunopositivity is not a prognostic factor in cHCC-CC; however, it is associated with α-fetoprotein, glypican 3 and EpCAM immunopositivity, indicating the mechanism of carcinogenesis. Further study is necessary to interpret the immunostaining pattern for SALL4.

Keywords: combined hepatocellular carcinoma and cholangiocarcinoma, EpCAM, immunohistochemistry, SALL4

Introduction

Combined hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC) (cHCC-CC) is a rare liver cancer containing unequivocal, intimately mixed

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elements of both HCC and CC. The reported prevalence rate varies from 0.87% to 6% of all primary liver cancers,[1,2] and the prognosis after surgical resection is unfavourable; a previous study showed a poorer prognosis than that of HCC.[2] Although the pathological and biological traits of cHCC-CC have not been fully elucidated, previous studies focused on tumour cells with a phenotype intermediate between hepatocytes and cholangiocytes, and the results indicated that cHCC-CCs are of hepatic stem/progenitor cell origin.[3,4]

The fourth edition of the World Health Organization (WHO) classification set up new entities named 'subtypes with stem-cell features', but the subtypes are not yet considered to be distinctive clinicopathological entities. This is because the biological differences are still uncertain, and studies of hepatic stem/progenitor cell markers in cHCC-CC are expected to identify the distinctive subtype.

Spalt-like transcriptional factor 4 (SALL4), the human homologue of the *Drosophila* spalt homeotic gene, which encodes a zinc finger transcription factor, is essential for the proliferation and stabilization of embryonic stem cells.[5] SALL4 regulates organogenesis, and it was reported that its functional role during liver development is to control the lineage commitment of hepatoblasts differentiating into hepatocytes or cholangiocytes.[6] SALL4 is expressed not only in fetal liver,[7] but also in various malignancies, such as acute myeloid leukaemia,[8] germ cell tumour,[9] gastric cancer,[10,11] and liver cancers, and it is known to be a novel stem/progenitor cell marker of the liver.[7] The prestigious work of Yong *et al.*[12] showed that SALL4 is a marker for a progenitor subclass of HCC with an aggressive phenotype, and that its expression has potential as a therapeutic target.

SALL4 expression in cHCC-CC has not been fully revealed so far. We therefore conducted a study of SALL4 immunopositivity in a large cohort of patients with cHCC-CC to clarify its relationship with clinicopathological features, based on the hypothesis that SALL4 could be a prognostic factor in cHCC-CC. We also analysed its relevance to immunopositivity for other stem cell/oncofetal markers of the liver, to clarify the meaning of SALL4 immunopositivity.

Materials and methods

CASE SELECTION

The present study was approved by the Kyushu University of Medical Human Investigation Committee (Institutional Review Board no. 26-232), and conformed to the ethical guidelines of the 1975 Declaration of Helsinki. For privacy protection, we removed all identifying information of the samples before analysis. We then reviewed the tissue sections of 3680 surgically resected primary liver cancers diagnosed at the Department of Anatomic Pathology of Kyushu University between 1981 and 2011. All slides were evaluated by three pathologists (Y.T., K.K., and S.A.) without knowledge of the clinical data, and the pathological diagnosis of cHCC-CC was performed according to the following criteria, based on the latest (4th) edition of the WHO classification: (i) tumours containing areas of typical HCC and areas of typical CC; or (ii) tumours containing uncertainly differentiated components (often referred to as mixed or intermediate between hepatocytes and cholangiocytes), regardless of whether a typical HCC and/or typical CC component was also present. We determined the tumour element as an HCC component, a CC component or an uncertainly differentiated component according to the morphology revealed by haematoxylin and eosin staining and the immunopositivity pattern shown by immunohistochemical stains: arginase-1, hepatocyte paraffin 1, α-fetoprotein (AFP), glypican 3, cytokeratin 19 (CK19), epithelial membrane antigen, and carbohydrate antigen 19-9. We regarded a component that showed gland or tubular structures morphologically and expressed biliary markers as a CC component, even if it lacked mucin production. We excluded the following types of tumour from the present study: (i) collision-type tumours; (ii) scirrhous-type HCCs; (iii) HCCs with only CK19 immunopositivity lacking ductal or tubular morphology; (iv) fibrolamellar HCCs; and (v) cholangiolocellular carcinomas without an HCC-like component. We then identified the 90 cases of cHCC-CC. The clinicopathological features of these 90 cases of cHCC-CC are summarized in Table 1. To compare the results with those for other primary liver cancers resected in the same period, we also obtained the tissue sections of 24 cases of hepatoblastoma, randomly selected 92 cases from 3352 HCCs, and randomly selected 67 cases from 314 intrahepatic CCs (ICCs).

IMMUNOHISTOCHEMISTRY

We performed immunohistochemical staining on the sections that had more than two components among HCC, CC and uncertainly differentiated components. For the tumour consisting of only an uncertainly differentiated component, we performed the same staining on the section that had only an uncertainly differentiated component. Immunohistochemical

Table 1. Clinicopathological features of the 90 combined hepatocellular carcinoma and cholangiocarcinoma cases

Characteristic	Value
Age (years), mean ± SD	62.0 ± 11.2
Sex (male/female), no. (%)	71:19 (78.9:21.1)
HBsAg-positive, no. (%)	38/89 (42.7)
HCV antibody-positive, no. (%)	29/88 (33.0)
Background liver, no.	
NL	9
CH	33
LF	18
LC	30
AFP ≥ 100 ng/ml, no. (%)	38/86 (44.2)
CEA ≥ 5.0 ng/ml, no. (%)	14/50 (28)
CA19-9 ≥ 37 U/ml, no. (%)	16/47 (34.0)
Tumour size (mm), mean ± SD	45 ± 28
Multiple tumours, no. (%)	32 (35.6)
Vascular invasion, no. (%)	49 (54.4)
Bile duct invasion, no. (%)	20 (22.2)
Lymph node metastasis, no. (%)	11 (12.2)
Distant metastasis, no. (%)	2 (2.2)
UICC stage, no.	
I	25
II	38
III	2
IVA	23
IVB	2

AFP, α-fetoprotein; CA19-9, Carbohydrate antigen 19-9; CEA, Carcinoembryonic antigen; CH, Chronic hepatitis; HBsAg, Hepatitis B surface antigen; HCV, Hepatitis C virus; LC, Liver cirrhosis; LF, Liver fibrosis; NL, Normal liver; SD, Standard deviation; UICC, Union for International Cancer Control.

staining was performed with the EnVision+ system (Dako, Glostrup, Denmark). The primary antibodies and methods used for antigen retrieval are shown in Table 2. After the inhibition of endogenous peroxidase and antigen retrieval, the sections were incubated with primary antibodies at 4°C overnight, and then with the secondary antibody/peroxidase-linked polymers. The sections were then treated with 3,3′-diaminobenzidine, and counterstained with haematoxylin. For each immunostaining procedure, a germ cell tumour of the testis was used as a positive control, and omission of primary antibody was used as a negative control. The EnVision G|2 Doublestain System (Dako) was used for double staining to evaluate coexpression of SALL4 and AFP, of SALL4 and glypican 3, and of SALL4 and epithelial cell adhesion molecule (EpCAM).

IMMUNOHISTOCHEMICAL EVALUATION

All slides were evaluated by three independent pathologists (Y.T., K.K., and S.A.). We defined immunostaining as positive if ≥5% of the tumour cells were stained with the proper reactive pattern for all antibodies.[12,13] Nuclear staining of SALL4 has been reported to show two patterns, i.e. a diffuse finely granular pattern, and a punctuate/clumped pattern;[14] however, it is unclear whether the punctuate/clumped pattern represents real expression of the hepatic stem cell/oncofetal marker. We regarded the diffuse finely granular pattern as immunopositive, based on Liu *et al.*[15], and evaluated whether there are any distinctive features regarding the cases with the punctuate/clumped pattern. We also evaluated the positivity for each HCC, CC and uncertainly differentiated component for comparison. The proportion of each component varied among the samples, and we therefore adopted another definition of positivity for the objective evaluation: If any positive cells were recognized in a component, the component was defined as immunopositive.

TAQMAN POLYMERASE CHAIN REACTION (PCR) TO DETECT THE QUANTITY OF SALL4 MRNA

We obtained 13 frozen samples of cHCC-CC. RNA extraction and quantitative real-time PCR for SALL4 was performed with TaqMan assay reagents (SALL4 Hs00360675_ml.; β-actin Hs99999903_ml.; Applied Biosystems, Foster City, CA, USA) as reported previously.[16] The obtained data were standardized with data of β-actin. All of the reactions for standard samples and samples of patients were performed in triplicate. The data were averaged from the values obtained in each reaction. The final numerical value (V) in each sample was calculated as follows: V = SALL4 mRNA value/β-actin mRNA value.

STATISTICAL ANALYSIS

The statistical analysis of group differences was performed with Fisher's exact test or the Mann-Whitney

Table 2. Primary antibodies used for immunohistochemical staining

Antibody	Source	Clonality	Dilution	Antigen retrieval
SALL4	Abnova, Taipei, Taiwan	6E3	1:1000	EDTA buffer (pH 8.0), pressure cooker for 20 min
AFP	Dako, Glostrup, Denmark	Polyclonal	1:400	Not done
Glypican 3	Bio Mosaics, Burlington, VT, USA	1G12	1:200	TRS buffer (pH 9.0), microwave for 20 min
EpCAM	Dako, Glostrup, Denmark	Ber-EP4	1:150	Trypsin, water bath for 5 min
NCAM	Leica Biosystems, Newcastle upon Tyne, UK	1B6	1:50	Citrate buffer (pH 6.0), microwave for 5 min
OV-6	R&D Systems, Minneapolis, MN, USA	OV-6	1:100	EDTA buffer (pH 8.0), microwave for 20 min
OCT-4	Santa Cruz Biotechnology, Santa Cruz, CA, USA	Polyclonal	1:500	EDTA buffer (pH 8.0), microwave for 30 min
NANOG	R&D Systems, Minneapolis, MN, USA	Polyclonal	1:1000	Citrate buffer (pH 6.0), pressure cooker for 5 min

AFP, α-fetoprotein; EpCAM, Epithelial cell adhesion molecule; NCAM, Neural cell adhesion molecule; SALL4, Spalt-like transcription factor 4.

U-test. A Kaplan-Meier analysis and the log rank test were used to derive and compare survival curves. A *P*-value of <0.05 was considered to indicate statistical significance. We carried out the analysis with JMP 11 (SAS Institute, Cary, NC, USA).

Results

SALL4 IMMUNOSTAINING PATTERN AND MRNA EXPRESSION DETERMINED WITH TAQMAN PCR

We found eight (8.9%) samples showing the diffuse finely granular immunostaining pattern and 20 (22.2%) showing the punctuate/clumped immunostaining pattern for SALL4 among 90 cHCC-CCs (Figure S1). There was no immunostaining in the background liver. SALL4 mRNA expression in three groups, according to the immunostaining pattern, was as follows: diffuse finely granular pattern ($n = 1$), mean value of 3.16×10^{-4}; punctuate/clumped pattern ($n = 3$), mean value of 72.9×10^{-4}; and no immunostaining ($n = 9$), mean value of 8.27×10^{-4}.

SALL4 IMMUNOPOSITIVITY AND THE DIFFERENCES BY THE COMPONENT OR THE DIFFERENTIATION OF THE TUMOUR

SALL4 was immunopositive in eight (8.9%) of 90 cHCC-CCs, and the range of immunopositivity was as follows: 5–9% of the tumour cells were immunopositive in two cases, 10–24% in two cases, 25–49% in two cases, and ≥50% in two cases. We morphologically and immunohistochemically identified 87 HCC components, 79 CC components and 61 uncertainly differentiated components in 90 cHCC-CCs. SALL4 was immunopositive in seven (8.0%; five moderately differentiated components and two poorly differentiated components) of the 87 HCC components, in five (6.3%; four moderately differentiated components and one poorly differentiated component) of the 79 CC components, and in six (9.8%) of the 61 uncertainly differentiated components (Figure 1A–F). There was no significant difference in the SALL4 immunopositivity rate among the three components. On comparison of SALL4 immunopositivity between the types of cHCC-CC according to the WHO classification, SALL4 was immunopositive in one (3.4%) of 29 classical types and in seven (11.5%) of 61 subtypes with stem-cell features. There were no significant differences between the two groups.

COEXPRESSION OF SALL4 AND OTHER STEM CELL/ONCOFETAL MARKERS

The association between SALL4 immunopositivity and other stem cell/oncofetal marker immunopositivity is shown in Table 3. Immunopositivity rates for EpCAM, AFP and glypican 3 in SALL4-immunopositive samples were significantly higher than those in SALL4-immunonegative samples ($P = 0.0004$, $P = 0.0478$, and $P = 0.0059$, respectively). Double staining showed SALL4-immunopositive cells with immunopositivity for EpCAM, AFP, and glypican 3 (Figure 1G–I).

ASSOCIATION BETWEEN SALL4 IMMUNOPOSITIVITY AND CLINICOPTHOLOGICAL FEATURES

We found no correlation between SALL4 immunopositivity and clinicopathological features (Table 4).

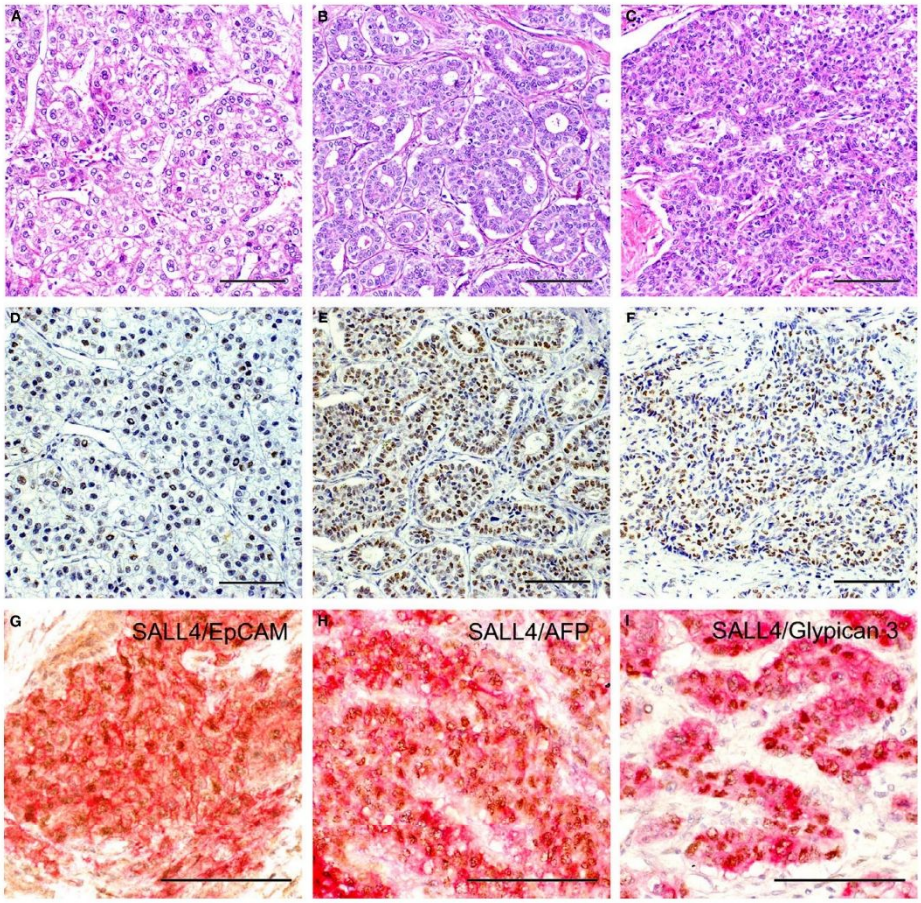

Figure 1. Microscopic findings in a single case of combined hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC). A–C, The tumour cells were proliferating in a trabecular pattern with clear cell change in the HCC component (A), in a tubular fashion in the CC component (B), and in a solid pattern with fibrous stroma in the uncertainly differentiated component (C). D–F, Spalt-like transcription factor 4 (SALL4) was immunopositive in all three components: the moderately differentiated HCC component (D), the well to moderately differentiated CC component (E), and the uncertainly differentiated component (F). G–I, Double staining of SALL4 (brown) and epithelial cell adhesion molecule (EpCAM) (red) (G), SALL4 (brown) and α-fetoprotein (AFP) (red) (H), and SALL4 (brown) and glypican 3 (red) (I). Double-positive cells were frequently seen. Scale bars: 100 μm.

There was also no significant difference in clinical outcome between the patients with and without SALL4 immunopositivity (Figure 2). None of the other stem cell/oncofetal markers showed a correlation between immunopositivity and clinical outcome.

SALL4 IMMUNOPOSITIVITY IN PRIMARY LIVER CANCERS

SALL4 was immunopositive in 11 (45.8%) of 24 hepatoblastomas, four (4.3%) of 92 HCCs, and three

Table 3. Association between spalt-like transcription factor 4 (SALL4) and other stem cell/oncofetal marker immunopositivity [no. (%)]

	SALL4-immunopositive (n = 8)	SALL4-immunonegative (n = 82)	P-value
AFP-positive	4 (50)	14 (17.1)	0.0478*
Glypican 3-positive	8 (100)	39 (47.6)	0.0059*
EpCAM-positive	8 (100)	28 (34.1)	0.0004*
NCAM-positive	2 (25)	27 (32.9)	1.0000
OV-6-positive	6 (75)	48 (58.5)	0.4684
OCT-4-positive	1 (12.5)	0 (0)	0.0889
NANOG-positive	0 (0)	0 (0)	1.0000

AFP, α-fetoprotein; EpCAM, Epithelial cell adhesion molecule; NCAM, Neural cell adhesion molecule.
*$P < 0.05$.

(4.5%) of 67 ICCs (Figure 3A–C). SALL4 immunopositivity was significantly more frequent in hepatoblastomas than in HCCs ($P < 0.0001$), cHCC-CCs ($P < 0.0001$), and ICCs ($P < 0.0001$) (Figure 3D). The HCC patients with SALL4 immunopositivity showed significantly poorer overall survival ($P < 0.0001$) and disease-free survival ($P < 0.0001$) (data not shown).

NUCLEAR PUNCTUATE/CLUMPED IMMUNOSTAINING PATTERN FOR SALL4

We found the nuclear punctuate/clumped immunostaining pattern for SALL4 in 20 (22.2%) of 90 cHCC-CCs. The punctuate/clumped immunostaining pattern was significantly more frequent in the HCC components than in the CC components ($P < 0.0001$) or uncertainly differentiated components ($P = 0.0024$) (Table S1). When we regarded both punctuate/clumped and diffuse finely granular patterns as immunopositive, the SALL4-immunopositive cases showed higher immunopositivity rates for AFP ($P < 0.0001$), glypican 3 ($P = 0.0058$), and EpCAM ($P = 0.0024$) (Table S2), a higher hepatitis B surface antigen positivity rate ($P = 0.0233$) and a higher serum AFP level (≥ 100 ng/ml) ($P = 0.0171$) (Table S3) than the SALL4-immunonegative cases. There was no significant difference in clinical outcome between the patients with and without SALL4 immunopositivity. Samples with the punctuate/clumped immunostaining pattern for SALL4 showed a significantly lower immunopositivity rate for EpCAM ($P = 0.0251$) than those with the diffuse finely granular pattern.

Table 4. Association between spalt-like transcription factor 4 (SALL4) immunopositivity and clinicopathological features

	SALL4-immunopositive (n = 8)	SALL4-immunonegative (n = 82)	P-value
Age (years), mean ± SD	62.0 ± 10.3	62.0 ± 11.3	0.9096
Sex (male/female)	4:4	67:15	0.0581
HBsAg-positive, no. (%)	5 (62.5)	33/80 (41.3)	0.2837
HCV antibody-positive, no. (%)	3 (37.5)	26/80 (32.5)	1.0000
AFP ≥ 100 ng/ml, no. (%)	6 (75)	32/78 (41.0)	0.1309
CEA ≥ 5.0 ng/ml, no. (%)	2/6 (33.3)	12/44 (27.3)	1.0000
CA19-9 ≥ 37 U/ml, no. (%)	0/5 (0)	16/42 (38.1)	0.1504
Tumour size (mm), mean ± SD	48 ± 15	45 ± 29	0.2390
Multiple tumours, no. (%)	2 (25)	30 (36.6)	0.7068
Vascular invasion, no. (%)	4 (50)	45 (54.9)	1.0000
Bile duct invasion, no. (%)	2 (25)	18 (22.0)	1.0000
Lymph node metastasis, no. (%)	0 (0)	11 (13.4)	0.5886

AFP, α-fetoprotein; CA19-9, Carbohydrate antigen 19-9; CEA, Carcinoembryonic antigen; HBsAg, Hepatitis B surface antigen; HCV, Hepatitis C virus; SD, Standard deviation.

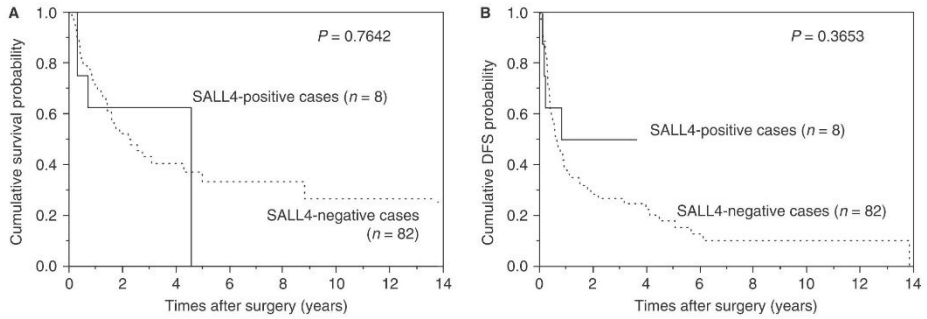

Figure 2. Overall survival (A) and disease-free survival (DFS) (B) curves by spalt-like transcription factor 4 (SALL4) immunopositivity in combined hepatocellular carcinoma and cholangiocarcinoma. There were no significant differences between the groups.

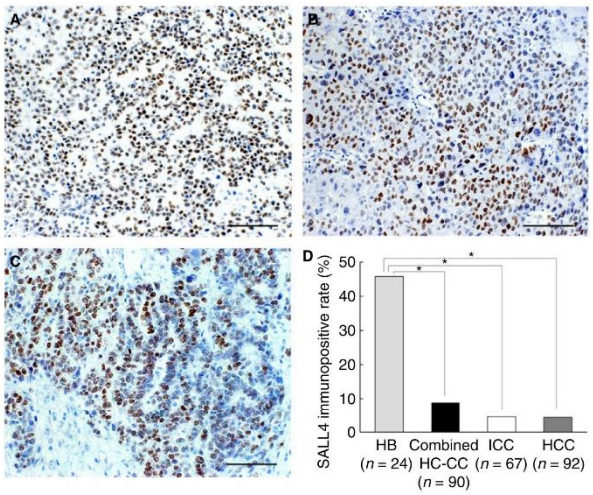

Figure 3. Immunohistochemical spalt-like transcription factor 4 (SALL4) staining in primary liver cancers. A–C, Immunopositivity for SALL4 was observed in hepatoblastoma (HB) (A), hepatocellular carcinoma (HCC) (B), and intrahepatic cholangiocarcinoma (ICC) (C). Scale bars: 100 μm. D, In our comparison of the SALL4 immunopositivity rate among the primary liver cancers, the highest rate was in HBs (45.8%), followed in order by combined HCC-cholangiocarcinomas (CCs) (8.9%), ICCs (4.5%), and HCCs (4.3%).

Discussion

This study demonstrates an association of SALL4 immunopositivity with EpCAM, AFP and glypican 3 immunopositivity in a large cohort of patients with cHCC-CC. This finding is very interesting from the perspective of the traits of SALL4-immunopositive tumour cells. Both AFP and glypican 3 are well known as oncofetal proteins and markers of HCC[17,18] and hepatoblastoma.[19] Hepatoblastoma is characterized by immature histology and a wide variety of cell lineages,[20] and we demonstrated here that the hepatoblastomas had the highest immunopositivity rate among the primary liver cancers. Furthermore, in

AFP-producing gastric carcinomas, it was shown that SALL4 immunopositivity was more frequent in the hepatoid areas than in the non-hepatoid areas, and that it was closely associated with immunopositivity for AFP and glypican 3.[10,11] Collectively, SALL4-immunopositive tumours may be associated with an immature stage of the hepatocyte lineage.

EpCAM is one of the hepatic stem/progenitor cell markers, and its expression is associated with invasiveness and tumorigenesis.[21] In addition, it was reported that SALL4 was activated in EpCAM-positive liver cancer stem cells, and the study's authors referred to the interaction between SALL4 and EpCAM, which regulates cell proliferation and the stemness of hepatoma cell lines.[22] Our data are consistent with those of the recent studies cited above. These results caused us to surmise that SALL4 and EpCAM interact with each other, and that this interaction may play an important role in the pathogenesis of cHCC-CC.

Our results were not concordant with the hypothesis that SALL4 immunopositivity may be associated with an unfavourable prognosis in cHCC-CC. The contradiction may be partly attributable to the fact that 79 (87.8%) of the 90 cHCC-CCs had CC components, which show a more aggressive behaviour and have a poorer prognosis than HCC.[2] Regarding the WHO classification, the SALL4 immunopositivity rates were not significantly different between the classical type and subtypes with stem-cell features; however, one possible reason for this is that we excluded cholangiolocellular carcinomas without HCC components from the present study.

Nuclear staining of SALL4 has been reported to show two patterns.[14] There are some reports regarding SALL4 immunopositivity in HCCs[7,12,14,15,22] and hepatoblastomas;[23] however, there is no rationale for positive staining of SALL4. A correlation between SALL4 gene and protein expression was demonstrated by Zeng *et al.*,[22] and here we confirmed SALL4 mRNA expression not only in samples showing a diffuse finely granular pattern but also in those showing a punctuate/clumped immunostaining pattern for SALL4. Although the SALL4 mRNA expression level in the diffuse finely granular pattern group was low, this could be because of the sampling lesion. Considering the stronger correlation with EpCAM immunopositivity, it is appropriate to define the diffuse finely granular pattern as immunopositive. However, from our data, we cannot definitively exclude the possibility that the punctuate/clumped immunostaining pattern represents SALL4 expression. A punctuate/clumped immunostaining pattern for SALL4 is frequently observed in HCC components, and this result is compatible with those reported by Gonzalez-Roibon *et al.*[14] When we defined both staining patterns as immunopositive, hepatitis B virus (HBV) infection and a high serum AFP level were associated with SALL4 immunopositivity. The association of SALL4 with HBV infection is in accord with several HCC studies.[12,22] Liu *et al.*[16] reported that there was no association between SALL4 immunopositivity and HBV infection in HCC; however, they specified that only the diffuse finely granular pattern was defined as immunopositive. HBV X protein was reported to activate EpCAM[24] and also to interact with histone deacetylase (HDAC).[25] HDAC is known to be involved in the pathway by which SALL4 represses phosphatase and tensin homologue,[26] and Zeng *et al.*[22] have suggested that SALL4 plays a role in controlling HDAC activity. There may be an association between HBV infection and SALL4 through the carcinogenesis process in cHCC-CCs. Further experiments with a larger number of samples are necessary to address this issue.

In conclusion, the present study provides novel findings of an association between SALL4 and clinicopathological features, particularly immunopositivity for AFP, glypican 3, and EpCAM. Furthermore, our findings reveal differences in nuclear staining patterns for SALL4, and indicate that further study is necessary for the interpretation of SALL4 immunostaining.

Acknowledgements

We appreciate the helpful comments and technical support from Dr Takeshi Iwasaki.

Author contributions

Y. Tanaka, S. Aishima and K. Kohashi designed and performed the research, performed slide review, analysed the data, and wrote the paper. H. Wang performed the immunohistochemical staining and PCR analysis. T. Hida performed the immunohistochemical staining. Y. Okumura and K. Kotoh analysed the data. K. Shirabe contributed to the collection of frozen samples and PCR analysis. R. Takayanagi and Y. Maehara contributed to the collection of samples and research design. Y. Oda designed the research and gave final approval of the manuscript. All authors critically reviewed and approved the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Microscopic findings of the diffuse finely granular pattern (A) and the nuclear punctuate/clumped pattern (B). Scale bars: 50 μm.

Table S1. Topographic status of immunoreaction with a punctuate/clumped immunostaining pattern.

Table S2. Association between spalt-like transcription factor 4 (SALL4) immunopositivity and clinicopathological features when we regarded both diffuse finely granular and punctuate/clumped patterns as immunopositive.

Table S3. Association between spalt-like transcription factor 4 (SALL4) and stem cell/oncofetal marker immunopositivity when we regarded both diffuse finely granular and punctuate/clumped patterns as immunopositive.

Living Donor Liver Transplantation for Intrahepatic Arteriovenous Fistula With Hepatic Artery Reconstruction Using the Right Gastroepiploic Artery

TO THE EDITOR:

Intrahepatic arteriovenous fistula (IHAVF), although extremely rare, is one of the indications for liver transplantation.[1] A representative congenital cause for IHAVF is hereditary hemorrhagic teleangiectasia or Rendu-Osler-Weber disease,[2] and the secondary causes are percutaneous puncture procedures such as liver biopsy.[3] Hepatic artery reconstruction in patients with IHAVF is considered difficult because the hepatic arteries usually enlarge due to high-output arterial flow into the fistula.[4] In living donor liver transplantation (LDLT), graft hepatic arteries are too thin to be directly anastomosed to these enlarged recipient hepatic arteries, and moreover, there is no vascular graft to be used as an interposition. Here, we report 2 cases of LDLT for IHAVF, in which hepatic artery reconstruction was accomplished using the recipient right gastroepiploic artery (RGEA).

Patient 1

Patient 1 was a 57-year-old woman who had undergone LDLT using a left lobe graft for primary biliary cirrhosis 10 years earlier. She had a history of having undergone liver biopsy for abnormal liver function tests. She presented with abdominal pain and tarry stool. The laboratory test results indicated that she had severe anemia. Contrast-enhanced computed tomography (CT) revealed that there was an intrahepatic arterioportal fistula in the liver graft and the portal venous system was immediately enhanced by contrast in the early phase (Fig. 1). Upper endoscopy showed risky gastric varices but no obvious bleeding point. No apparent bleeding point could be detected by total colonoscopy. Capsule endoscopy finally detected multiple oozing points in the small intestine. These results indicated that severe portal hypertension led to the intestinal bleeding. Angiography confirmed the presence of an IHAVF between the intrahepatic segment 3 arteries and portal veins (PVs) and that the inflow hepatic artery was enormously enlarged. We tried to obstruct the IHAVF by interventional radiology to no avail, because of the multiple intrahepatic collateral arterial flows. Eventually, liver transplantation became the only option for curing the intestinal bleeding. Subemergency living donor liver retransplantation was performed using a left hepatic graft donated from her son 3 months after the presentation of abdominal pain and tarry stool. The pretransplant liver function tests are summarized in Table 1. Upon dissecting around the hepatic hilum, the enlarged hepatic artery whose diameter was approximately 1 cm was encountered, as preoperatively expected. Because of the size discrepancy

Abbreviations: CT, computed tomography; HA, hepatic artery; IHAVF, intrahepatic arteriovenous fistula; LDLT, living donor liver transplantation; LHA, left hepatic artery; LHV, left hepatic vein; MHV, middle hepatic vein; PV, portal vein; RGEA, right gastroepiploic artery.

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Received September 10, 2015; accepted November 6, 2015.

View this article online at wileyonlinelibrary.com.

DOI 10.1002/lt.24377

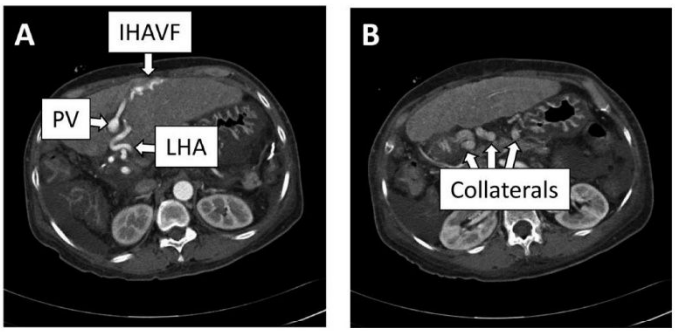

FIG. 1. Contrast-enhanced CT of patient 1. (A) An IHAVF presumably caused by the liver biopsy was noted in segment 3. The LHA was tortuous and enlarged. The PV in the umbilical portion was immediately enhanced in the early phase. (B) Tortuous collaterals were noted around the stomach.

TABLE 1. Preoperative and Current Liver Function Tests of the 2 Patients

		Patient 1		Patient 2	
Item	Normal Range	Preoperative	Current	Preoperative	Current
White blood cell (/μL)	3300-8600	6740	6370	5810	7000
Hemoglobin (g/dL)	11.6-14.8	10.6	12.9	10.1	12.5
Platelet $\times 10^3$ (/μL)	158-348	228	213	359	397
Albumin (g/dL)	4.1-5.1	2.6	3.9	2.9	3.9
Total bilirubin (mg/dL)	0.4-1.5	3.7	0.8	1.2	0.5
Creatinine (mg/dL)	0.46-0.79	0.74	0.71	0.44	0.57
Aspartate aminotransferase (U/L)	13-30	126	25	53	48
Alanine aminotransferase (U/L)	7-23	83	24	45	37
Alkaline phosphatase (U/L)	106-322	504	341	1901	645
Prothrombin time–international normalized ratio	0.90-1.10	1.00	Not determined	1.28	Not determined

between the graft and recipient hepatic arteries, it was impossible to directly anastomose these arteries. The hepatic vein and the PV were reconstructed in the usual manner. The recipient RGEA was freed from the greater curvature of the stomach by ligating the small branches into the stomach, and the RGEA (2 mm in outer diameter) was anastomosed to the graft left hepatic artery (LHA; 2 mm in outer diameter; Fig. 2A,B).[5] Doppler ultrasound examination confirmed excellent arterial wave forms (Fig. 2C). The arterial flow measured by a flow meter was 144 mL/minute, which was considered sufficient in an LDLT with a left hepatic graft. The biliary reconstruction was accomplished by a hepaticojejunostomy. The postoperative course was uneventful without any ischemic conditions of the graft liver. For 6 years after the retransplantation, she had been constantly in a good condition. The contrast-enhanced CT taken on 3 years after the retransplantation showed the intact arterial flow from the recipient RGEA to the graft LHA. The current liver function tests (6 years after the retransplantation) of this patient were excellent (Table 1).

Patient 2

Patient 2 was a 43-year-old female. She had been suffering from multiple intrahepatic secondary biliary stenoses caused by multiple arteriovenous malformations for 6 years before the transplantation. Interventional biliary stenting was initially effective for relieving the biliary strictures. Cholangitis and intrahepatic abscesses, however, had gradually become refractory. Contrast-enhanced CT revealed that there were enormously enlarged hepatic arteries (Fig. 3). Then, she had begun to complain of exertional dyspnea caused by high-output cardiac failure approximately 3 years before the transplantation. There was no detectable arteriovenous malformation other than the liver. Liver transplantation was the last resort against these

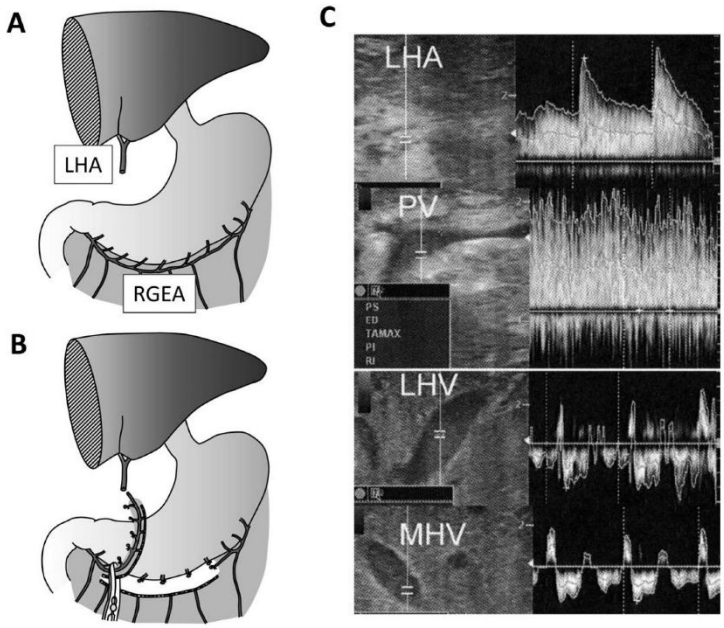

FIG. 2. (A and B) Schematic diagrams of the preparation of the RGEA for hepatic artery reconstruction and (C) Doppler ultrasound wave forms of the intrahepatic hepatic artery, PV, LHV, and MHV of patient 1. (A) First, the dividing point of the RGEA was determined by measuring the distance between the stump of the LHA and the root of the RGEA so that there could remain some redundancy after anastomosis. (B) The RGEA was freed from the greater curvature of the stomach by dividing the omentum below the RGEA using a vessel sealing device and by ligating and dividing the small branches into the stomach. The root of the RGEA was clamped and then the RGEA was divided. The stump of the RGEA was brought up via the antegastric route without kinking of the artery. (C) Doppler ultrasound examination confirmed excellent arterial wave forms.

deteriorating conditions. Her husband wished to donate his partial liver, and the patient was referred to our hospital to undergo LDLT. Preoperative 3D-CT of the donor revealed that the left hepatic graft would have 2 small hepatic arteries (the left and middle hepatic arteries). The pretransplant liver function tests are summarized in Table 1. On entering the peritoneal cavity, tangled, enormously enlarged hepatic arteries were encountered in the hepatoduodenal ligament. These arteries together with the extrahepatic bile duct were divided at the hepatic hilum using an autosuture device. The hepatic vein and the PV were reconstructed in the usual manner. The hepatofugal portal venous flow spontaneously returned to hepatopetal one after reperfusion. The recipient RGEA was freed from the greater curvature of the stomach by ligating the small branches into the stomach, and the RGEA (2 mm in outer diameter) was anastomosed to the graft LHA (2 mm in outer diameter). The arterial flow measured by a flow meter was 88 mL/minute, which was considered adequate in an LDLT with a left hepatic graft. The graft middle hepatic artery (1.5 mm in diameter) was ligated after confirmation that there was sufficient backflow from the stump of the middle hepatic artery. Biliary reconstruction was done by a hepaticojejunostomy. Her postoperative course was uneventful without any ischemic conditions of the graft liver. For 6 months after the transplantation, she had been in a good condition. The contrast-enhanced CT taken 1 month after the transplantation showed the

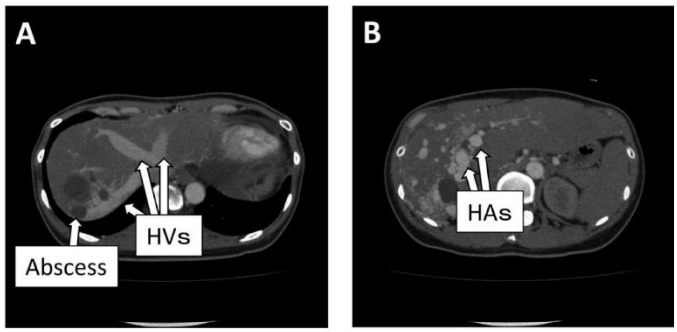

FIG. 3. Contrast-enhanced CT of patient 2. (A) The intrahepatic veins were vigorously enhanced even in the very early phase of contrast-enhanced CT. (B) The hepatic arteries enormously enlarged to nearly the size of the aorta.

intact arterial flow from the recipient RGEA to the graft LHA. The current liver function tests (6 months after the transplantation) were satisfactory (Table 1).

In considering LDLT for IHAVF, the most important fact that surgeons have to pay attention to is that the recipient hepatic arteries have to be enormously enlarged to be directly anastomosed to the graft hepatic arteries. The hepatic arteries in patients with IHAVF have excessive blood flow because they are directly connected to low-pressure venous systems. As a result, the hepatic arteries gradually enlarge, and by the time liver transplantation is considered, they become too large to be directly anastomosed to tiny graft arteries. Unlike deceased liver transplantation, the graft hepatic arteries to be reconstructed in LDLT were too thin to be directly anastomosed to those huge hepatic arteries in patients with IHAVF, and there is usually no interposition vascular graft obtained from deceased donors. The usefulness of the recipient RGEA for hepatic artery reconstruction has been reported by many investigators.[5] The RGEA has great flexibility compared to other visceral arteries such as the left gastric artery or the splenic artery. The normal RGEAs, however, are usually thin and their diameters are approximately less than 2 mm. Surgeons may think that the arterial flow of the RGEA is not sufficient to provide adequate arterial blood through the entire hepatic graft. However, there have not been any reports suggesting ischemic conditions related to hepatic artery reconstruction by the RGEA although the number of patients with such reconstruction was small. Most patients with IHAVF have undergone multiple sessions of interventional therapy because certain portions of IHAVFs are interventionally treated. As a result, the inflow hepatic artery may be injured and may not be suitable for an inflow artery for hepatic artery reconstruction. Hepatic artery reconstruction using the left gastric artery or the splenic artery was reported with success.[4,5] However, it is generally difficult to dissect the left gastric artery free from the lessor curvature of the stomach due to the massive collateral vessels. Moreover, it is not always possible to obtain enough length of the left gastric artery so as to be directly anastomosed to the graft artery. On the other hand, dissecting the splenic artery from the retroperitoneum is far more difficult.

We determined to use a left graft in the 2 patients although they were an adult-to-adult LDLT. Generally, patients with IHAVF who need liver transplantation have relatively good hepatic reserve. Therefore, a small left hepatic graft can be selected in these patients. At this point, a question arises. Can the recipient RGEA be used safely even in LDLT using a larger right hepatic graft? We strongly believe the arterial flow supplied by the RGEA can at least maintain the graft viability as long as the anastomosis remains intact.

From these points of view, the RGEA can be the first choice for an inflow artery in recipients with IHAVF. Surgeons should ascertain that there is the intact RGEA in the recipient during preoperative evaluation for patients with IHAVF.

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