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EBAG9/RCAS1 expression in hepatocellular carcinoma: correlation with tumour dedifferentiation and proliferation

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Abstract

The oestrogen-responsive gene, *EBAG9*, whose product is identical to the cancer cell surface antigen RCAS1, is reported to be associated with tumour progression and invasiveness in various carcinomas. In this study, we examined the expression of EBAG9/RCAS1 in hepatocellular carcinoma (HCC), with special reference to its relationship with the stepwise evolution of HCC. Expression was examined by immunohistochemistry and western blotting analysis in 143 HCCs, as well as in non-cancerous liver tissues. After which, the association between enhanced EBAG9/RCAS1 expression and various clinicopathological parameters including Ki-67 labelling index (LI), a marker of proliferative activity, was evaluated. There was a constant low level of EBAG9/RCAS1 expression in non-cancerous liver tissues, with a regular cytoplasmic distribution. Positive immunoreactivity for EBAG9/RCAS1 was detected on the surface and in the cytoplasm of 84 HCC tumours, with an irregular staining pattern. Enhanced EBAG9/RCAS1 expression was correlated with a lower degree of differentiation and Ki-67 LI. Interestingly, expression was enhanced specifically in the less differentiated lesions within 'nodule-in-nodule' tumours. In conclusion, EBAG9/RCAS1 was associated with HCC tumour dedifferentiation and increased proliferative activity. Its exact functional role remains to be established.

Keywords: EBAG9/RCAS1; Hepatocellular carcinoma; Immunohistochemistry; Western blotting analysis; Tumour dedifferentiation; Tumour proliferation; Ki-67 labelling index

1. Introduction

ER-binding fragment-associated antigen 9 (EBAG9) was originally identified as a human oestrogen-responsive gene in a cDNA library of MCF-7 human breast cancer cells, and its mRNA was shown to be directly upregulated by oestrogen [1]. By preparing a rabbit polyclonal antibody against EBAG9, we showed that it is upregulated by oestrogen *in vivo* in the mouse uterus, and that it is also expressed in various other mouse tissues, including the liver [2]. It has been reported recently, in a study using the same antibody, that EBAG9 is also widely distributed in human breast carcinomas [3].

Receptor-binding cancer antigen expressed on SiSo cells (RCAS1), which was isolated as the antigen recognised by 22-1-1 antibody against a human uterine adenocarcinoma cell line SiSo [4], has been shown to be identical to EBAG9 (EBAG9/RCAS1) [5]. It has received attention as a potential cancer-associated antigen in various organs [4,6–16]. Expression is generally thought to be related to tumour invasiveness and also to be associated with a poor patient prognosis. It has been suggested that the close relationship between EBAG9/RCAS1 expression and tumour invasiveness may be accounted for by the *in vitro* observation that this protein facilitates escape from host immune surveillance by inducing apoptosis of activated $CD3^+$ T lymphocytes and NK cells [5].

Hepatocellular carcinoma (HCC) has the unique characteristic that its development and progression

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exhibits a typical multistep pathology [17]. In general, most early HCCs [18,19] are small (<2 cm in diameter), well-differentiated nodules with a low proliferative activity, and their size remains unchanged for months or, in some cases, years. When they progress to a more advanced stage, they transform into moderately- to poorly-differentiated cancers, accompanied by a rapid increase in size. During this process (tumour dedifferentiation and proliferation), the HCCs also increase in malignant potential, as reflected in intrahepatic metastasis (IM) and vascular invasion (VI). This transformation occurs non-uniformly within a given tumour nodule, resulting in the coexistence of well-differentiated and moderately- to poorly-differentiated lesions within the same nodule. This has been termed by histologists a 'nodule-in-nodule' or 'mosaic' appearance [17].

It was previously reported that RCAS1 expression could not be detected in normal liver samples probed with 22-1-1 antibody, but was found in approximately one quarter of HCC cases, particularly in patients with VI of the tumour [16]. Expression of EBAG9/RCAS1 has, however, not yet been investigated in detail in relation to the multistep progression of HCC. In the present study, we have investigated the association of its expression with the successive stages of HCC progression by immunohistochemistry and western blot analysis utilising a rabbit anti-EBAG9 polyclonal antibody. We paid particular attention to (1) the process of tumour dedifferentiation, (2) cancer proliferative activity, and (3) ability to metastasise. We evaluated cancer proliferative activity by the Ki-67 labelling index (LI), which has been used as a marker of proliferation in various cancers including HCC [20,21]. We also examined the correlation between EBAG9/RCAS1 immunoreactivity and various clinical parameters, including disease-free survival.

2. Patients and methods

2.1. Study population and follow-up of patients

From October 1994 to December 1998, 232 radical hepatic resections were performed for HCC in the Hepato-Biliary-Pancreatic and Transplantation Division, Department of Surgery, Tokyo University Hospital, Tokyo. Among them, 58 of the patients received repeat hepatectomies for recurrent tumours, and 6 underwent hepatic resection for mixed HCC and cholangiocarcinoma [22], and these cases were excluded from the study population. In addition, 25 patients were excluded because of totally necrotised tumours caused by preoperative transcatheter arterial embolisation or chemo-lipiodolisation. The 143 patients enrolled included 109 males and 34 females, with a mean age of 62.0±11.0 (mean±standard deviation (S.D.)) years (range 13–83 years). 23 patients were positive for hepatitis B surface antigen (HBs Ag), and 100 for anti-hepatitis C virus antibody (HCV Ab). One patient was positive for both.

At hepatectomy, resected liver specimens were fixed in 10% v/v formalin, cut into blocks, and embedded in paraffin. They were then sliced into 4-μm sections, and stained with haematoxylin and eosin, or used for immunohistochemical analysis. A part of each tumour, as well as adjacent non-cancerous tissue, was snap frozen in liquid nitrogen and stored at −80 °C for later protein isolation.

Normal liver tissue was obtained for the immunohistochemical analysis from 10 potential liver transplant donors, who provided liver biopsy specimens for preoperative evaluation. Ten liver biopsies carried out for the assessment of serologically HCV Ab positive patients were also made available for analysis.

Follow-up data were obtained from medical records or telephone interviews and were available in 142 cases. They were updated for patient's disease-free survival to 30 June 2002 (mean follow-up period 45.5 months, range 6.3–85.8 months). The patients received monthly abdominal ultrasonography and their levels of alpha-fetoprotein (AFP) (normal range <20 ng/ml) and des-γ-carboxy prothrombin (DCP) (normal range <62.5 AU/ml) were measured. In addition, they underwent dynamic computed tomography (CT) at least every 6 months. Angiography with lipiodol injection was added when recurrence was suspected and recurrence was assessed by a combination of these imaging modalities.

2.2. Serology

EBAG9 antibody was a rabbit polyclonal antibody against a glutathione-S-transferase (GST)-EBAG9 fusion protein [2]. Its specificity has been examined previously in Ref. [3]; in summary, it was shown to react with human and mouse EBAG9, yielding a band of 32kD in western blotting analysis, and the intensity of the band was reduced by prior incubation of the antibody with recombinant EBAG9 protein. Monoclonal antibody for Ki-67 (MIB 1) and monoclonal antibody for β-actin (Clone AC-74) were purchased from DAKO (Carpinteria, CA, USA) and Sigma-Aldrich (Saint Louis, MI, USA), respectively.

2.3. Immunohistochemistry

Tissue sections of each HCC nodule and adjacent non-cancerous liver tissue, as well as the liver biopsy specimens, were submitted to immunohistochemical analysis. Where there were multiple nodules the largest nodule was considered to be representative. After removing paraffin in xylene, and rehydrating in a graded ethanol series, the sections were processed in 10 mM

citrate buffer (pH 6.0) and heated in an autoclave at 125 °C for 5 min for antigen retrieval. Endogenous peroxidase activity was quenched with 3% v/v hydrogen peroxidase/methanol. The slides were preincubated with 10% v/v fetal calf serum-phosphate buffered saline at room temperature to reduce non-specific antibody binding. They were then incubated overnight at 4 °C with the EBAG9 antibody diluted 1:40, or with the Ki-67 antibody diluted 1:50. Immunostaining was performed by a peroxidase labelled-dextran polymer method using EnVision + ™ (DAKO, Carpinteria, CA, USA). Staining was visualised with 3,3′-diaminobenzidine tetrahydrochloride working solution (Funakoshi, Tokyo, Japan), with counterstaining in Mayer's haematoxylin. For negative controls, primary antibody was replaced with normal rabbit (diluted 1:800) or mouse (diluted 1:100) IgG (DAKO, Carpinteria, CA, USA). Sections of human breast cancer that had been shown to be EBAG9-positive were processed as positive controls.

Scoring of Ki-67 in HCCs was performed on high-power fields (×400) by light microscopy. In each case, more than 500 carcinoma cell nuclei were counted independently by two of the authors, and the percentage of immunoreactive cells, that is, LI, was determined. Final scores were established by consensus.

2.4. Western blotting analysis

Western blotting was performed on representative samples of HCC and non-cancerous liver tissue. Frozen sections of five HCC tissues and adjacent non-cancerous liver tissues (all histologically diagnosed as cirrhotic) were homogenised on ice in RIPA buffer (50 mM Tris–HCl (pH 8.0), 200 mM NaCl, 20 mM NaF, 2 mM ethyleneglycol tetraacetic acid, 1 mM dithiothreitol, 2 mM sodium vanadate, 0.5% v/v Nonidet P-40 supplemented with protease inhibitor cocktail (Complete™; Boehringer Mannheim GmbH, Mannheim, Germany)). The homogenates were clarified by centrifugation at 12 000*g* for 30 min at 4 °C. Samples of lysates containing 8 μg protein [23] were boiled for 5 min in the presence of β-mercaptoethanol. They were then fractionated on 10–20% w/v sodium dodecyl sulphate (SDS)-polyacrylamide gradient gel and transferred electrophoretically onto a polyvinylidene difluoride membrane (Fluorotrans, Japan Genetics, Tokyo, Japan) using a buffer consisting of 25 mM Tris, 192 mM glycine, and 20% v/v methanol. The membrane was blocked at room temperature for 30 min in Tris-buffered saline with 1% v/v Tween 20 (TBS-T) and 5% w/v skimmed milk, then incubated overnight at 4 °C with rabbit anti-EBAG9 serum diluted 1:1000 or mouse monoclonal antibody against β-actin diluted 1:2500. The membrane was washed in TBS-T, and incubated with 1:5000 diluted horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Pharmatica Biotech, Tokyo, Japan) at room temperature for 1 h. After further washings, the protein–antibody complexes were visualised with the enhanced chemiluminescence (ECL™) plus detection system (Amersham Pharmatica Biotech, Tokyo, Japan) according to the manufacturer's protocol. A whole cell lysate of a human prostate cancer cell line, DU145, was used as a positive control.

2.5. Analysis of the association between EBAG9 expression and clinicopathological variables

The correlation between EBAG9 expression and various clinical and pathological parameters was analysed using the Chi-square or Mann–Whitney U test, as appropriate. Seven clinical and seven pathological variables were examined as follows: patient age (65 years versus $\geqslant$65 years), gender, HBs Ag, HCV Ab, Child–Turcotte–Pugh score (Grade A versus B) [24], serum AFP level, plasma DCP level, degree of tumour differentiation (well versus moderately or poorly), size of largest tumour nodule ($\leqslant$2.0 versus $>$2.0), tumour number (solitary versus multifocal), microscopic IM and/or microscopic VI, fibrous capsular formation/infiltration, pathology of non-cancerous liver tissue (fibrosis versus chronic hepatitis versus cirrhosis) and Ki-67 LI. The presence of microscopic IM and/or microscopic VI was clustered into a single variable and defined as tumour invasiveness [25]. When a tumour nodule contained components of different grades of differentiation, the tumour was classified according to the predominant grade. A P value of 0.05 was considered statistically significant.

To detect factors associated with patient's disease-free survival, univariate analysis was performed using Cox proportional hazards model for EBAG9 or other clinicopathological variables, and the risk ratio and 95% Confidence Intervals (CI) were calculated [26]. Data were analysed using the StatView 5.0J software (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. EBAG9 expression in normal and chronically diseased liver tissue

Non-cancerous hepatocytes, including those from normal, chronic HCV-related hepatitic or cirrhotic tissues, displayed a low, but significant, level of EBAG9 immunoreactivity compared with negative controls (Fig. 1a–e). High power magnification (×1000) revealed punctate staining throughout the cytoplasm (Fig. 1c).

3.2. EBAG9 expression in HCCs

HCC cells showed variable EBAG9 immunoreactivity. Some had baseline weak immunoreactivity

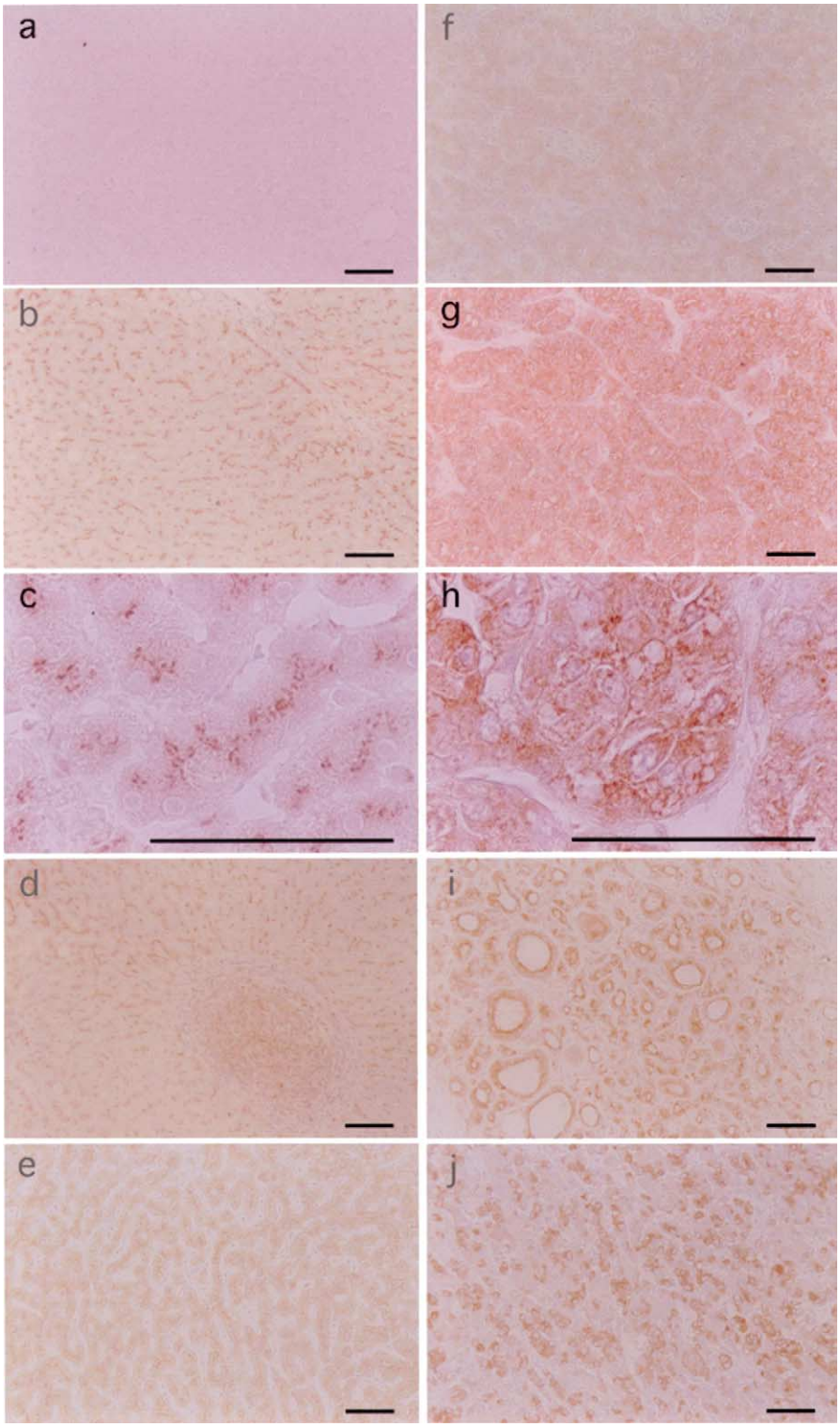

Fig. 1. Expression of ER-binding fragment-associated antigen 9 (EBAG9) in non-cancerous liver tissues (a–e) and hepatocellular carcinomas (HCCs) (f–j). (a) Negative controls showing no immunoreactivity (×200). (b–e) Normal liver tissue (b), chronic hepatitic tissue (d), and cirrhotic tissue (e) showing weak immunoreactivity (×200). A regular distribution of weakly stained dots is seen in the cytoplasm of normal hepatocytes (c) (×1000). (f) A well-differentiated HCC classified as intensity-negative. The pattern of expression is similar to that in non-cancerous tissues (×200). (g and h) An intensity-positive case of moderately-differentiated HCC (trabecular type) (g) (×200). Immunoreactivity is detected over the entire surface and in the cytoplasm of the cancer cells. Coarse, thickened granules are dispersed in the cytoplasm, and the regularity of the staining pattern is lost (h) (×1000). (i) Pseudoglandular type of moderately-differentiated HCC displaying intense expression on the apical surface of the cells (×200). (j) A poorly-differentiated HCC consisting of small cells with enhanced EBAG9 expression on their surface and in the cytoplasm (×200). Bar: 20 μm.

similar to non-cancerous hepatocytes. These were designated intensity-negative cells. Others showed enhanced immunoreactivity, and were designated intensity-positive. With regard to staining pattern, intensity-negative cancer cells had a similar pattern to non-cancerous hepatocytes, namely, a regular distribution of weakly stained granules throughout the cytoplasm (Fig. 1f). In contrast, in most of the intensity-positive cells, there was intense staining over the entire surface, as well as in the cytoplasm (Fig. 1g–j). Coarse, thickened granules were dispersed throughout the cytoplasm, and the regularity of the granule distribution noted in the non-cancerous hepatocytes was lost (Fig. 1h). In the pseudoglandular type of moderately differentiated HCCs, the apical surfaces of the pseudoglands were strongly stained (Fig. 1i).

The proportion and distribution of intensity-positive cancer cells were highly variable from nodule to nodule (range 5–100%). Interestingly, their distribution also showed two types of intranodular diversity: (1) 'Nodule-in-nodule' tumours, i.e. those consisting of a well-differentiated lesion and a less differentiated lesion, displayed different immunoreactivity in the two regions, the less differentiated intensely immunoreactive region contrasting clearly with the weakly immunoreactive well-differentiated region (Fig. 2a). (2) The peripheral zone of HCC nodules with fibrous capsular infiltration stained strongly at the site of infiltration (Fig. 2b).

3.3. Semiquantitative classification of EBAG9 expression in HCCs

We next evaluated the expression of EBAG9 in HCC tissues in a semiquantitative manner. We classified sections into three categories: (1) Negative (−): sections in which all the cancerous cells were identified as intensity-negative; (2) borderline (±): sections where 1–5% of the malignant cells stained for EBAG9, or, sections showing uniform positivity but very weak immunoreactivity; (3) positive (+): sections with more than 5% of positive cancerous cells. The classification of the sections was carried out in a blinded manner and finally agreed upon by two of the authors. In the event, 35 of the 143 lesions examined (24%) were classified as negative, 24 (17%) as borderline, and 84 (59%) as positive (Table 1).

3.4. Western blotting analysis

The results of the western blotting analysis are shown in Fig. 3. All the non-cancerous tissues expressed EBAG9 as a 32-kD band. Expression of EBAG9 was not enhanced in two of the five cancerous tissues examined (cases 1 and 2: one well-differentiated and one moderately-differentiated HCC); however, it was enhanced in the remaining three samples (cases 3–5: moderately-differentiated HCCs). The division of HCCs by western blotting analysis into those in which expression of EBAG9 was enhanced, and those in which it was not, agreed well with the classification into intensity-positive/-negative nodules by semiquantitative immunohistochemistry.

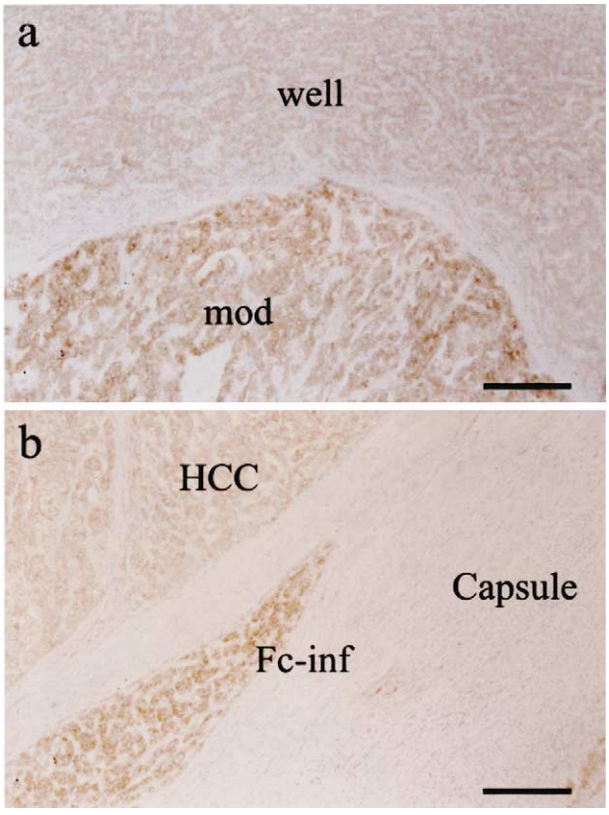

Fig. 2. Distribution of intensity-positive cancer cells in hepatocellular carcinomas (HCC) nodules: (a) a tumour with 'nodule-in-nodule' appearance showing intense staining in the interior moderately-differentiated region ('mod'), contrasting with weak staining in the outer, well-differentiated, region ('well') (×100); (b) a moderately-differentiated HCC showing fibrous capsular infiltration ('Fc-inf') with enhanced staining at the site of infiltration (×100). Capsule: fibrous capsule. Bar: 50 μm.

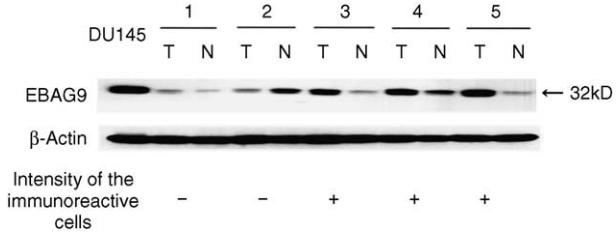

Fig. 3. Western blotting analysis. All non-cancerous liver samples (N) express ER-binding fragment-associated antigen 9 (EBAG9) as a 32-kD band. Expression of EBAG9 is not enhanced in two of the hepatocellular carcinomas (HCCs) (1 and 2), but is enhanced in the other 3 (3–5). The results of the western blotting analysis are consistent with the classification based on the immunohistochemistry. Western blotting using monoclonal anti-β-actin antibody demonstrated that equal amounts of protein were used. T, HCC tissue; N, non-cancerous liver tissue; kD, kilodalton.

Table 1
Frequency distribution of immunocytochemical staining of HCCs for EBAG9

Classification of staining	Notation	Number of HCC tumours	Percentage of HCC tumours
Negative	−	35	24
Borderline	±	24	17
Positive	+	84	59
Total		143	100

HCC, Hepatocellular carcinoma; EBAG9, ER-binding fragment-associated antigen 9.

3.5. Correlation between EBAG9 expression and pathological variables

For the purpose of analysis, unless otherwise specified, the borderline staining HCCs were combined with the unstained tumours into one group, leaving the clearly positive staining HCCs as the other group. The relationship between EBAG9 immunoreactivity and clinicopathological variables was then analysed by comparing the intensity-positive group ($n=84$) with the intensity-negative/borderline group ($n=59$), as shown in Table 2. Enhanced EBAG9 immunoreactivity was more frequently observed in the less differentiated tumours ($P=0.01$). In addition, EBAG9 immunoreactivity was significantly related to Ki-67 LI (Fig. 4). In the intensity-negative/borderline group, the mean Ki-67 LI was 16.4% (median 12.6, range 0.5–56.0) whereas it was 24.8% in the intensity-positive group (median 23.8, range 2.9–68.6) ($P<0.001$). However, there was no significant correlation between enhanced EBAG9 expression and tumour invasiveness (IM and/or VI) ($P=0.86$). Other clinical and pathological variables were also not significantly associated with enhanced EBAG9 expression, although the relationship between enhanced EBAG9 expression and elevated plasma DCP value was of borderline significance ($P=0.1$).

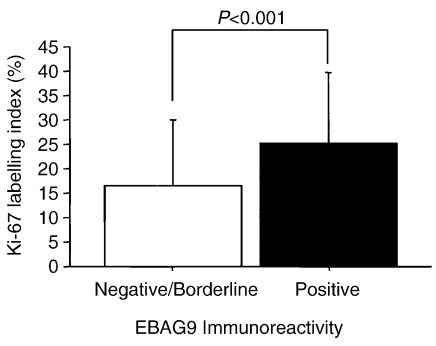

Fig. 4. Association of ER-binding fragment-associated antigen 9 (EBAG9) expression with Ki-67 labelling index (LI).

3.6. Relationship between EBAG9 expression and disease-free survival

During the follow-up period, HCC recurrence was confirmed in 107 patients. On the univariate analysis, plasma DCP level, tumour size, tumour number, tumour invasiveness (IM and/or VI), and Ki-67 LI (>20 versus $\leqslant 20$) were significantly related to the patient's disease-free survival (Table 3). However no significant relationship was established between enhanced EBAG9 expression and disease-free survival ($P=0.17$).

4. Discussion

In the present study, we have investigated the expression of EBAG9/RCAS1 in various HCC and non-HCC liver tissues, including those from normal livers and those chronically infected with HCV and/or HBV. We performed a comprehensive immunohistochemical analysis using a specific antibody against EBAG9 which is also suitable for western blotting analysis, paying special attention to the association of EBAG9 expression with tumour dedifferentiation, proliferation and metastasis.

EBAG9/RCAS1 was expressed, albeit weakly, in both normal and chronically diseased non-cancerous hepatocytes. In addition, it showed a regular pattern of expression within the cytoplasm, suggesting that it becomes localised in some way. The finding of EBAG9/RCAS1 in non-cancerous hepatocytes contradicts the results of a previous report [16]. However, it is consistent with the detection of EBAG9/RCAS1 expression in a number of non-cancerous human organs, e.g., uterine endometrial glands [27], goblet cells of bronchi and bronchioles [7,8], mammary glands [3], and gastric mucosa [10]. It is notable that the apical surfaces of the pseudoglands were strongly stained in the pseudoglandular type of moderately-differentiated HCCs, as shown in Fig. 1i. This observation, together with previous results obtained from normal tissues, suggests that EBAG9/RCAS1 is expressed in gland cells.

In this study, 59 and 17% of the patients examined showed positive and borderline expression of EBAG9/RCAS1, respectively. This roughly agrees with the proportion found in other malignancies (47% in lung cancers to 70% in gallbladder cancers) [8,11]. However, Noguchi and colleagues reported that only 26.5% of HCC cases expressed RCAS1 compared with the 59% showing positivity for EBAG9 reported above [16]. We can be confident that our immunohistochemical staining for EBAG9 is specific for this protein for the following reasons: (a) The specificity of the anti-EBAG9 polyclonal antibody has been established by the preabsorption test reported previously in Ref. [3]. (b) In western

Table 2
Association between EBAG9 immunoreactivity and clinicopathological parameters

Parameters	Number of patients	Intensity of EBAG9 immunoreactivity		*P* value
		Negative/borderline (%)	Positive (%)	
		62.2±11.6	61.8±10.6	0.44
Age (years)[a]				
<65	75	28 (37)	47 (62.7)	
⩾65	68	31 (46)	37 (54.4)	0.39
Gender				
Male	107	40 (37)	67 (62.6)	
Female	36	19 (53)	17 (47.2)	0.12
HBs Ag				
+	23	10 (43)	13 (56.5)	
−	120	49 (41)	71 (59.2)	0.82
HCV Ab				
+	100	42 (42)	58 (58.0)	
−	43	17 (40)	26 (60.5)	0.85
Child–Turcotte–Pugh score				
A	110	43 (39)	67 (60.9)	
B	33	16 (48)	17 (51.5)	0.42
AFP (ng/ml)[b]		51 (2–436 000)	55 (2–69 000)	0.57
⩽62.5	48	17 (35)	31 (65)	
>20	95	42 (44)	53 (56)	0.37
DCP (AU/ml)[b]		62.5 (11–80 000)	76.0 (10–77 520)	0.10
⩽6.25	76	36 (47)	40 (53)	
>62.5	67	23 (34)	44 (66)	0.12
Degree of tumour differentiation				
Well	25	16 (64)	9 (36)	
Moderately/poorly	118	43 (36)	75 (64)	0.01
Tumour size (cm)[a]		2.8 (0.8–15.5)	3.4 (1.0–16.0)	0.29
⩽2.0	38	20 (53)	18 (47)	
>2.0	105	39 (37)	66 (63)	0.12
Tumour number				
Solitary	79	35 (44)	44 (56)	
Multifocal	64	24 (38)	40 (63)	0.49
IM and/or VI				
Positive	58	23 (40)	35 (60)	
Negative	85	36 (42)	49 (58)	0.86
Fibrous capsular formation/infiltration				
Positive/positive	79	30 (38)	49 (62)	
Positive/negative	27	13 (48)	14 (52)	
Negative	37	16 (43)	21 (57)	0.62
Background liver disease				
Liver fibrosis	5	1 (20)	4 (80)	
Chronic hepatitis	40	13 (33)	27 (68)	
Liver cirrhosis	98	45 (46)	53 (54)	0.21
	143	59 (46)	84 (59)	

EBAG9; ER-binding fragment-associated antigen 9; HBs Ag, HBs Ag-positive; HCV Ab, HCV Ab-positive; AFP, alpha-fetoprotein; DCP, des-β-carboxy prothrombin; IM, intrahepatic metastasis; VI, vascular invasion.
[a] Data expressed as average±standard deviation (S.D.).
[b] Data expressed as median value (range).

blots, the EBAG9 antibody recognised a protein forming a specific 32-kD band, consistent with the size of human EBAG9. (c) The intensity of the 32-kD band agreed with the semiquantitative classification of EBAG9 immunoreactivity by immunohistochemistry. Therefore, the fact that the proportion of EBAG9-positive patients was almost as twice as high in our series is not the result of non-specific staining. It may be due to the different antibodies and methods used in the two studies.

Table 3
Clinical risk factors for disease-free survival ($n = 142$)

Parameters	Categories (number of patients)	Risk ratio	95% CI	*P* value
Age (years)	$\geqslant 65/< 65$ (68/74)	0.88	0.60–1.28	0.51
Gender	Male/female (106/36)	0.92	0.60–1.43	0.74
HBs Ag	Positive/negative (23/119)	0.76	0.44–1.32	0.33
HCV Ab	Positive/negative (100/42)	1.02	0.67–1.56	0.91
Child–Turcotte–Pugh score	B/A (33/109)	1.05	0.66–1.66	0.83
AFP (ng/ml)	$> 20/\leqslant 20$ (94/48)	1.08	0.72–1.62	0.68
DCP (AU/ml)	$> 62.5/\leqslant 62.5$ (66/76)	1.63	1.11–2.39	0.01
Degree of tumour differentiation	Moderately + poorly/well (117/25)	0.99	0.60–1.63	0.97
Tumour size (cm)	$T > 2.0/T \leqslant 2.0$ (104/38)	1.87	1.17–3.00	0.008
Tumour number	Multifocal/solitary (64/78)	1.80	1.22–2.64	0.002
IM and/or VI	Positive/negative (57/85)	1.67	1.13–2.46	0.009
Fibrous capsular formation	Positive/negative (106/36)	1.05	0.67–1.63	0.82
Ki-67 LI (%)	$> 20/\leqslant 20$ (70/72)	1.63	1.11–2.39	0.01
EBAG9 immunoreactivity	Positive/negative + borderline (83/59)	1.30	0.88–1.92	0.17

EBAG9; ER-binding fragment-associated antigen 9; HBs Ag, HBs Ag-positive; HCV Ab, HCV Ab-positive; AFP, alpha-fetoprotein; DCP, des-g-carboxy prothrombin; IM, intrahepatic metastasis; VI, vascular invasion; 95% CI, 95% Confidence Interval.

Another finding of the present study is that in the majority of HCC tissues that showed enhanced EBAG9/RCAS1 expression, expression was detected over the entire surface and cytoplasm of the cancer cells, rather than displaying the restricted distribution observed in non-cancerous tissues. This finding is consistent with observations on invasive ductal carcinoma cells of the breast, where it was found that normal mammary gland cells only expressed EBAG9/RCAS1 on their apical surface, whereas carcinoma cells showed enhanced expression without a polar distribution [3].

Of the various clinicopathological parameters, enhanced expression of EBAG9/RCAS1 was closely related to degree of tumour differentiation and increased Ki-67 LI. Ki-67 antigen is preferentially expressed during all active phases of the cell cycle (G_1, S, G_2 and M phases), but is absent in resting cells [28]. In addition, the Ki-67 LI has been reported to be well correlated with the uptake of bromodeoxyuridine (BrdU), a well accepted proliferation-associated marker [29,30]. Since Ki-67 staining does not require the *in vivo* injection of a specific agent, which is necessary in BrdU studies, Ki-67 is now widely used as a marker of cell proliferation including in human studies [31]. Therefore, our observation suggests that enhanced EBAG9/RCAS1 expression is associated with HCC tumour progression as represented by dedifferentiation and proliferation. Interestingly, tumours that showed a 'nodule-in-nodule' appearance displayed a variable degree of EBAG9/RCAS1 expression that depended on the degree of differentiation within the tumour, i.e. intense expression in the less differentiated regions and weak expression in the more highly differentiated regions (Fig. 2a). Similarly, lesions characterised by fibrous capsular infiltration had intense EBAG9/RCAS1 staining at the site of infiltration (Fig. 2b). These findings lend support to the link between the level of EBAG9/RCAS1 expression and tumour progression.

In contrast, EBAG9/RCAS1 was not associated with tumour metastasis (IM and/or VI) in our series, although a significant relationship between RCAS1 expression and VI was reported previously in Ref. [16]. All these results led us to conclude that EBAG9/RCAS1 is closely associated with tumour dedifferentiation and proliferation, but not in tumour metastasis, at least in resectable HCCs. In other words, EBAG9/RCAS1 presumably relates more to the growth of the primary tumour than to the development of tumour metastases. This view may be partially supported by an idea proposed recently on the basis of the results of cDNA microarray analysis, namely that different genes function at different stages of HCC evolution [32]. According to this view, HCC requires additional steps with further discrete genetic changes, i.e. dedifferentiation and proliferation, to gain the ability to metastasise. Our results may therefore imply that enhanced EBAG9/RCAS1 expression is an intermediate event in the multistep progression of HCC, unrelated to the final event characterised by the frequent occurrence of vascular invasion and resultant intrahepatic metastasis. In our series, there was no significant difference in disease-free survival between patients with positive and negative/borderline EBAG9 immunoreactivity. The results are consistent with the finding that enhanced EBAG9 expression was not associated with metastatic parameters such as IM and VI, which have been the strong factors predictive of poor prognosis in the previous studies [33,34]. Taken together, EBAG9 may not be a prognostic factor in patients with resectable HCC, however, we believe it is of value as a pathological marker of a specific stage of HCC tumour progression.

In conclusion, we observed weak, but discretely localised, expression of EBAG9/RCAS1 in non-cancerous normal and chronically diseased liver tissues, suggesting that EBAG9/RCAS1 is expressed in a positionally regulated fashion. We further found that the expression of this protein was enhanced in approximately half of the HCCs, and that this enhanced expression was characterised by loss of the localised staining pattern. Enhanced EBAG9/RCAS1 expression was correlated with tumour dedifferentiation and proliferation, but not with metastasis. Future investigation of the role of EBAG9/RCAS1 in non-cancerous and cancerous liver tissues should help to clarify the mechanism of HCC progression.

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