Prognostic Significance and Functional Relevance of Olfactomedin 4 in Early-Stage Hepatocellular Carcinoma

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OBJECTIVES: **Hepatocellular carcinoma (HCC) is a leading cancer-related cause of death. Unfortunately, recurrence is common even after curative treatment of early-stage patients, and no adjuvant treatment has yet been established. Aberrant expression of OLFM4 in human cancers has been reported; yet, its specific function during tumor development remains poorly understood, and its role in HCC is unknown. The purpose of this study is to examine the prognostic significance of OLFM4 and its functional relevance in determining recurrence in patients with early-stage HCC.**

METHODS: **Immunohistochemical staining to assess expression, cellular distribution, and prognostic significance of OLFM4 was performed in a tissue microarray comprising 157 HCC tissues and matched nontumor tissues. In addition, expression of OLFM4-coding mRNA was assessed in a separate patients' cohort. The findings were validated by *in vitro* functional studies using siRNA directed against OLFM4 to assess its effect on cell motility and proliferation.**

RESULTS: **The fraction of HCC samples exhibiting positive OLFM4 staining was higher in comparison with that observed in hepatocytes from matched nontumor tissue (61% vs 39%). However, cytoplasmic-only staining for OLFM4 was associated with vascular invasion ($P = 0.048$), MMP-7 expression ($P = 0.002$), and poorer survival ($P = 0.008$). A multivariate analysis confirmed the independent significance of OLFM4 in determining patients' outcome (5-year survival [58.3% vs 17.3%; HR: 2.135 {95% confidence interval: 1.135–4.015}; $P = 0.019$]). Correspondingly, inhibition of OLFM4 by siRNA modulated the expression of MMP-7 and E-cadherin, causing inhibition of cell proliferation, motility, and migration.**

DISCUSSION: **To the best of our knowledge, we provide the first report on the prognostic significance of OLFM4 in HCC and identify its mechanistic role as crucial mediator of MMP family protein and E-Cadherin in determining cell invasion and metastasis formation.**

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related death worldwide, with approximately 800,000 people dying each year because of this tumor (1,2). In early-stage disease, HCC can be treated in curative intention by surgery, local ablative procedures, or liver transplantation (3). Unfortunately, recurrence is common even in selected patients and, so far, no adjuvant therapeutic schema proved effective in delaying the time to recurrence (4).

Although HCC is a very heterogeneous tumor, and a multiplicity of molecular targets have been proposed, no biomarker-driven or stage-specific systemic treatment is available. To identify the factors contributing to determine tumor relapse after curative treatment might help identify high-risk patients who could benefit from a closer follow-up after curative treatment. In addition, the identification of markers of recurrence might also provide information on the crucial factors involved in tumor development and progression and help identify novel possible targets for adjuvant treatment.

Olfactomedin 4 (OLFM4, also known as GW112 or hGC-1) is a glycoprotein found in different tissues, comprising the bone marrow, the gastrointestinal tract, and the prostate (5). Although OLFM4 is involved in the physiological development of tissues and inflammation, overexpression of OLFM4 has been found in numerous solid neoplasms, including gastric (6), colorectal (7),

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pancreatic (8), lung, and breast cancer (9), as well as in leukemia cells (10). Although some works have identified the overexpression of OLFM4 as a distinctive feature of early-stage tumor development (9,11,12), the precise role of OLFM4 in carcinogenesis seems to be dependent on the tumor entity and the stage of tumor development. Interestingly, increased levels of OLFM4 could also be detected in the serum of patients with gastric cancer, in whom the concentration of OLMF4 proved higher than that in nontumor patients (13), suggesting that OLFM4 could be used as a circulating tumor biomarker (14,15).

In addition to these *ex-vivo* studies, functional experiments reinforced the notion of OLFM4 playing a role in cancer formation by showing that OLFM4 determines cell motility and metastasis formation, as exemplified by the fact that high expression levels of OLFM4 causes decrease of adhesion and increase of migration in the colon cancer cells (15). In addition, OLFM4 may affect cell proliferation and cell death because it was shown to attenuate apoptosis by blocking caspase 3 and caspase 9 in gastric and prostate cancer cells (16,17).

Although these data point to a role of OLFM4 in cancer development, the relevance of this molecule in the pathogenesis of HCC has not yet been investigated. We thus examined the expression and cellular distribution of OLFM4 in HCC tissues and matched nontumour tissues and performed silencing experiments *in vitro* to assess the prognostic significance of this molecule and its functional role in determining recurrence of HCC.

MATERIALS AND METHODS

Patients and pathologic material

Patients with HCC who underwent liver transplantation or partial liver resection at the University Hospital, LMU Munich, during the period of 1985 and 2015 were considered for the study. To avoid a potential bias related to the exceptionally favorable prognosis of patients undergoing liver transplantation, analysis of survival was performed separately for patients who received liver transplantations and patients who underwent partial liver resection. Data on survival were obtained from the Munich Cancer Registry (http://www.tumorregister-muenchen.de). Archival pathological material was obtained from the Institute of Pathology of the University of Munich. The characteristics of the tissue microarray-containing tumor samples and matched tumor tissues have been previously described (18).

Immunohistochemical staining

Sections (5 μm) of tissue microarray blocks were used for immunohistochemical staining. Anti-OLFM4 polyclonal rabbit antibody (BIOZOL GmbH, Eching, Germany), anti-MMP-7 monoclonal mouse antibody (Merck KgaA, Gernsheim, Germany), and anti-E-cadherin monoclonal mouse antibody (Cell Signaling Technology, Danvers, MA) were applied as primary antibodies. The samples have been processed for antigen retrieval as previously described (19). The Vectastain ABC Elite Universal (Vector Laboratories, Burlingame, CA) kit was used for immunohistochemical staining; AEC (Zytomed Systems, Berlin, Germany) was used as a chromogen. Positive staining for OLFM4 was categorized according to its cellular distribution and independently of total staining intensity according to the following categories: cytoplasmic staining, staining on cell membranes only, and positive staining signals in both cytoplasm and cell membrane. In addition, we conducted semiquantitative analyses by categorizing the samples according to the percentage of cells exhibiting staining for OLFM4: score 0, no staining; score 1, staining in <30% of cells; score 2, staining in 30%–70% of cells; and score 3, staining in >70% cells. Concerning OLFM4 cytoplasmic staining, score 0 represented negative staining; score 1 represented weak staining, <70% of cells; and score 2 represented strong staining, >70% of cells.

Histologic assessment of tumors and surrounding nontumor tissues

Tumor grading according to the WHO criteria (20), presence of vascular invasion, number and size of tumor lesions (as defined by macroscopic inspection of surgical specimens) were analyzed as tumor-associated pathological variables. Matched tissues not containing tumor tissue were analyzed for features related to the underlying liver disease and comprised the presence and quantification of fibrosis/cirrhosis, portal inflammation, piecemeal necrosis, and steatosis. Histologic evaluation was performed on haematoxylin/eosin-stained slides and evaluated by a senior pathologist and 2 of the authors (L.K. and E.N.D.T.) who had no previous knowledge of the prognostic data. The Ishak score was used to evaluate liver fibrosis, portal inflammation, and piecemeal necrosis (21). The nonalcoholic fatty liver disease activity score and staging system was used for assessment of steatosis and lobular infiltration (22).

Cell culture

Huh7 and PLCPRF5 cells (ATCC, Old Town Manassas, VA) were cultured in Dulbecco's Modified Eagle Medium (Sigma-Aldrich, Taufkirchen, Germany), with 10% fetal bovine serum (Biochrom GmbH, Berlin, Germany) and 1% Penicillin-Streptomycin (Sigma-Aldrich). Cells were maintained in 5% CO_2 at 37 °C. Authentication of cell lines was conducted by Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures.

Western blot

Equal amount of proteins in each sample was loaded on 10% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and separated for 25 minutes at 80 V and for 80 minutes at 120 V and then transferred onto polyvinylidene difluoride membranes (EMD Millipore, Burlington, MA). After blocking for 1 hour in Tris-buffered saline with Tween 20 containing 5% milk or 5% bovine serum albumin (Carl Roth, Karlsruhe, Germany), the membranes were incubated overnight at 4 °C with the following primary antibodies: OLFM4, MMP-2 (Cell Signaling Technology, Danvers, MA), MMP-7, MMP-9 (Cell Signaling Technology), E-cadherin, and β-actin (Cell Signaling Technology). Subsequently, the membranes were probed with horseradish peroxidase-conjugated secondary antirabbit or antimouse IgG antibodies (GE Healthcare UK Limited, Buckinghamshire, UK) for 2 hours at room temperature in the dilution of 1:10,000. The bands were visualized by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Buckinghamshire, UK) and photographed with an image acquisition system, ECL ChemoCam Imager (Intas GmbH, Göttingen, Germany).

siRNA interference

Cells were plated to reach a confluence of 40%–60%. After overnight incubation, cells were transfected using Oligofectamine (Invitrogen, Karlsruhe, Germany) and siRNA directed against OLFM4 (siRNA-1, sense: GAGUUAACCUGACCACCAATT, antisense: UUGGUGGUCAGGUUAACUCTG. siRNA-2, sense: GGGAUUCUUUGUACAGGAATT, antisense: UUCCUGUACAAAGAAUCCCTA. Qiagen, Düsseldorf, Germany) or with beta-galactosidase (Dharmacon, Lafayette, CO), which served as

Table 1. Summary of clinical and pathologic features

Feature	Patient count N	%
Age at diagnosis (y)		
<60	70	44.6
≥60	87	55.4
Sex		
Male	122	77.7
Female	35	22.3
Etiology		
HCV	42	26.8
HBV	16	10.1
Toxic/metabolic	92	58.6
Unknown	7	4.5
Severe fibrosis/cirrhosis		
<5	64	40.8
≥5	88	56.0
Not available	5	3.2
Steatosis		
0	65	41.4
1–3	87	55.4
Not available	5	3.2
Portal inflammation		
0–2	71	45.2
3–4	81	51.6
Not available	5	3.2
Piecemeal necrosis		
0–2	119	75.8
3–4	33	21.0
Not available	5	3.2
Lobular inflammation		
0–1	113	72
2–3	39	24.8
Not available	5	3.2
Tumor size (cm)		
<5	81	51.6
≥5	76	48.4
Extrahepatic metastasis		
No	151	96.2
Yes	6	3.8
Grading		
1	29	18.5
2	81	51.6
3	33	21.0
Not available	14	8.9
Blood vessel invasion		
No	122	77.1
Yes	35	22.9
Multifocal lesions		
No	106	67.5
Yes	47	29.9
Not available	4	2.6
E-cadherin		
0–1	21	13.4
2–3	132	84.1
Not available	4	2.5
MMP-7		
0–1	27	17.2
2–3	129	82.2
Not available	1	0.6
Treatment		
Liver transplantation	56	35.7
Partial hepatectomy	101	64.3

the control at a final concentration of 50 nM. Serum-containing medium was added 4 hours after transfection. Silencing of OLFM4 was confirmed by immunoblotting 24 hours after transfection.

Immunofluorescence

Seventy-five thousand cells/well were plated on round cover slips (Thermo Scientific, Rockford, IL) with a diameter of 18 mm in 6-well plates (NUNC, Langenselbold, Germany). Twenty-four hours after transfection with siRNA, the cells were fixed in paraformaldehyde (4%, Carl Roth) for 15 minutes, treated with 0.15% Triton X-100 (Sigma-Aldrich, Taufkirchen, Germany) for 15 minutes in PBS (Invitrogen, Karlsruhe, Germany), and then blocked for 30 minutes with 5% BSA in PBS. The samples were incubated with antibodies against OLFM4 and E-cadherin diluted in blocking solution at 1:200 at 4 °C overnight and after washing, subsequently incubated for 1 hour with goat antirabbit antibody conjugated with Alexa Fluor 488 (Invitrogen) at 1:200 dilution. After 3 washes with PBS, the slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA) containing Hoechst 33342 (Sigma-Aldrich). Pictures were taken using an Axiovert 135 TV fluorescence microscope (Zeiss, Oberkochen, Germany).

Flow cytometry

Sub-G1 events and cell cycle distribution were assessed to measure apoptosis and different phases of cell cycle by using a fluorescence-activated cell sorter, an Accuri C6 Flow Cytometer, and BD Accuri C6 software (BD Biosciences, Germany). Cells were seeded in 6-well plates to reach a confluence of 50%–60% before undergoing transfection with siRNA. After 24 hours, the cells were split by trypsin (Invitrogen), collected and washed with sterilized ice-cold PBS once and then incubated in staining buffer

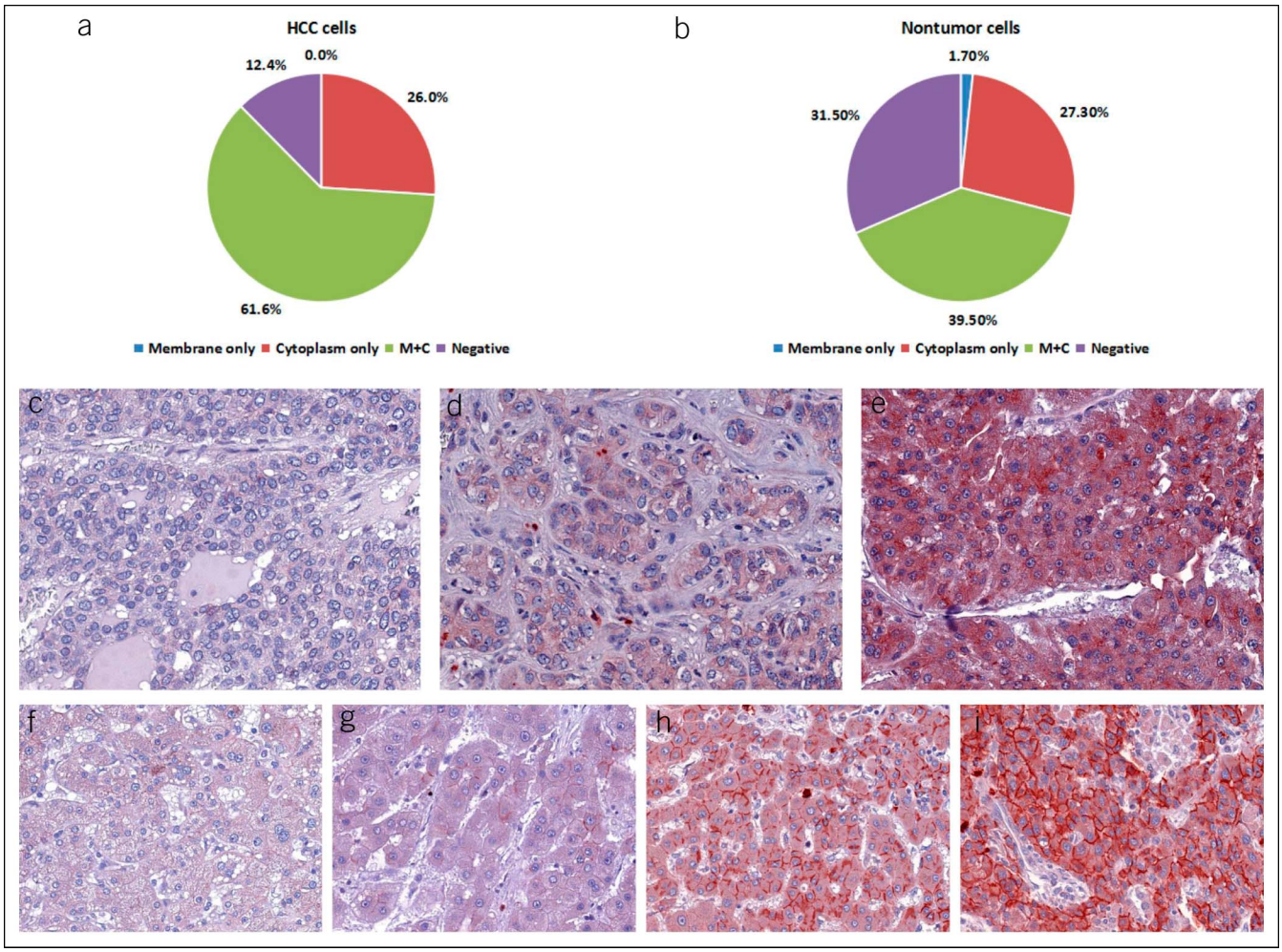

Figure 1. Expression and cellular distribution of OLFM4 in tumor and nontumor tissue samples. (**a**, **b**). Staining of OLFM4 in HCC cells and in nontumor cells: proportion of samples showing no staining (none), cytoplasmic staining (cytoplasm only), membrane staining (membrane only), or both (m + c). Representative cytoplasm staining pattern in HCC samples: (**c**) score 0, negative; (**d**) score 1, weak; (**e**) score 2, strong. Representative typical positive membrane staining in HCC tissues: (**f**) score 0, negative; (**g**) score 1, less than 30% of cells; (**h**) 30%–70% of cells; (**i**) over 70% of cells. Magnification, ×40. HCC, hepatocellular carcinoma.

containing 0.1% sodium citrate (Carl Roth), 0.1% Triton X-100, and 50 μg/mL propidium iodide (PI; Sigma-Aldrich).

Proliferation assay

Thousand to 1,500 cells were seeded in 96-well plates (NUNC), cultured overnight, and then incubated with siRNA. After 6 days, the cells were washed with PBS and underwent osmotic lysis in 100 μL ddH_2O for 45 minutes at 37 °C. 0.2% Sybr green (Lonza, Köln, Germany) was added to each well, fluorescence was measured, and proliferation index was calculated as a ratio to untreated samples. Three independent experiments were performed per agent, with each data point reflecting triplicate wells. Error bars represent SD of the mean from 3 experiments.

Scratch assay

Cells were seeded in 6-well plates to reach a confluence of 80% and treated as indicated in the siRNA section. After cells became confluent, a scratch was made by a sterile micropipette tip (Sarstedt AG, Nümbrecht, Germany). The scratch area was photographed after the scratch was performed and after 24 hours to assess cell migration within the wounded area. Migration was quantified by measuring the area of the scratched regions by ImageJ software (National Institutes of Health, Bethesda, Maryland, https://imagej.nih.gov/ij/). The experiment was performed thrice.

Migration and invasion assay

The chamber of 8-μm transwell inserts (Corning, NY), with or without Matrigel (Corning), was used for migration and invasion assay. 2×10^5 cells underwent incubation in the serum-free top chamber while serum-containing medium was added to the lower chamber. To assess the fraction of cells that migrated, staining with 1% crystal violet (Sigma-Aldrich) was performed after 24 hours of incubation and fixation in 10% formalin (Carl Roth). For microscopy, cells were evaluated at a magnification of ×200 (Zeiss, Germany).

Cancer genome atlas analyses

Publically available RNA sequencing data of HCC samples (N = 423) were downloaded from the Cancer Genome Atlas (TCGA)

Table 2. Correlation between OLFM4 staining and clinicopathologic parameters in HCC cells

Feature	Cytoplasm staining			Membrane staining		
	Negative, N (%)	Positive, N (%)	*P*	Negative, N (%)	Positive, N (%)	*P*
Age at diagnosis (y)						
<60	11 (7.0)	59 (37.6)	0.316	28 (17.8)	42 (26.8)	0.680
≥60	9 (5.7)	78 (49.7)		32 (20.4)	55 (35.0)	
Sex						
Male	13 (8.3)	109 (69.4)	0.144	45 (28.7)	77 (49.0)	0.522
Female	7 (4.5)	28 (17.8)		15 (9.6)	20 (12.7)	
Etiology						
HCV	4 (2.7)	38 (25.3)	0.684	21 (14.0)	21 (14.0)	0.236
HBV	2 (1.4)	14 (9.3)		7 (4.7)	9 (6.0)	
Toxic/metabolic	14 (9.3)	78 (52.0)		32 (21.3)	60 (40.0)	
Severe fibrosis/cirrhosis						
<5	11 (7.2)	53 (34.9)	0.232	27 (17.8)	37 (24.3)	0.559
≥5	9 (5.9)	79 (52.0)		33 (21.7)	55 (36.2)	
Steatosis						
0	13 (8.6)	52 (34.2)	0.05	31 (20.4)	34 (22.4)	0.052
1–3	7 (4.6)	80 (52.6)		28 (18.4)	59 (38.8)	
Portal inflammation						
0–2	8 (5.3)	63 (41.4)	0.633	22 (14.5)	49 (32.2)	0.088
3–4	12 (7.9)	69 (45.4)		36 (23.7)	45 (29.6)	
Piecemeal necrosis						
0–2	16 (10.5)	103 (67.8)	0.842	48 (31.6)	71 (46.7)	0.456
3–4	4 (2.6)	29 (19.1)		11 (7.2)	22 (14.5)	
Lobular inflammation						
0–1	7 (4.6)	106 (69.7)	<0.001[a]	43 (28.3)	70 (46.1)	0.811
2–3	13 (8.6)	26 (17.1)		14 (9.2)	25 (16.4)	
Tumor size (cm)						
<5	10 (6.4)	71 (45.2)	0.879	34 (21.7)	47 (29.9)	0.317
≥5	10 (6.4)	66 (42.0)		26 (16.6)	50 (31.8)	
Extrahepatic metastasis						
No	18 (11.5)	133 (84.7)	0.169[b]	59 (37.6)	92 (58.6)	0.408[b]
Yes	2 (1.3)	4 (2.5)		1 (0.6)	5 (3.2)	
Grading						
G1	2 (1.4)	27 (18.9)	0.367[b]	13 (9.2)	16 (11.1)	0.289
G2 and G3	18 (12.6)	96 (67.1)		39 (27.3)	75 (52.4)	
Blood vessel invasion						
No	19 (12.1)	103 (65.6)	0.048[a,b]	45 (28.7)	77 (49.0)	0.522
Yes	1 (0.6)	34 (21.7)		15 (9.6)	20 (12.7)	
Multifocal lesions						
No	15 (9.8)	91 (59.5)	0.552	42 (27.5)	64 (41.8)	0.686
Yes	5 (3.2)	42 (27.5)		17 (11.1)	30 (19.6)	
E-cadherin						
0–1	3 (1.9)	18 (11.8)	0.74	12 (7.8)	9 (5.9)	0.042[a]
2–3	17 (11.1)	115 (75.2)		45 (29.4)	87 (56.9)	

Table 2. (*continued*)

Feature	Cytoplasm staining			Membrane staining		
	Negative, N (%)	Positive, N (%)	*P*	Negative, N (%)	Positive, N (%)	*P*
MMP-7						
0–1	9 (5.8)	18 (11.5)	0.002[a,b]	16 (10.3)	11 (7.0)	0.015[a]
2–3	11 (7.1)	118 (75.6)		44 (28.2)	85 (54.5)	
Treatment						
Liver transplantation	11 (7.0)	45 (28.7)	0.053	27 (17.2)	29 (18.5)	0.055
Partial hepatectomy	9 (5.7)	92 (58.6)		33 (21.0)	68 (43.3)	

HCC, hepatocellular carcinoma.
[a]Statistical significance.
[b]Fisher exact test. All other *P* values were calculated by Pearson's χ^2 test.

(http://cancergenome.nih.gov/). The analyses of fragments per kilobase of transcript per million fragments values of genes in TCGA HCC patient samples and survival outcome were conducted as previously described (23).

Statistical analysis

All statistical analyses were calculated using SPSS (version 24; SPSS). For frequency data, exact χ^2 tests were used. Differences between groups were calculated by using the Student *t*-test. Overall survival was estimated with the Kaplan-Meier method and tested with the log-rank procedure. For analysis of survival, a Cox proportional-hazards regression model was used and reported by hazard ratios (HRs) and 95% confidence intervals (CIs). Statistical significance was set for a P value < 0.05.

RESULTS

Patients and clinicopathological parameters

One hundred fifty-seven patients with HCC included in a tissue microarray of specimens from patients with HCC undergoing liver resection or liver transplantation were considered for clinicopathological analysis. To avoid biases related to the effect of transplantation on patients' outcome, 56 patients who had undergone liver transplantation were not considered for survival analysis, which was performed in 95 of 101 patients with available survival data. The demographic and clinicopathologic characteristics of the patients in our cohort are presented in Table 1. For patients included in the survival analysis, the median follow-up time was 22 months. The median follow-up for survivors was 42 months vs 17 months for deceased patients.

Immunohistochemical staining for OLFM4 in HCC cells and in hepatocytes from surrounding nontumor tissues

In nontumor tissue samples, OLFM4 stained positive in altogether 68.5% of cases, showing a pattern of mixed membrane and cytoplasmic staining in 39.5% of patients (Figure 1), cytoplasmic-only staining in 27.3% of patients, and a membrane-only staining in 1.7% of the samples. In 31.5% of the samples from nontumor tissues, no staining for OLFM4 could be detected. On the other hand, altogether 87.6% showed a positive staining for OLFM4 in HCC: the frequency of cytoplasmic staining (26% of samples) was similar to that observed in nontumor tissues, whereas the fraction of patients with staining on both membranes and cytoplasm was 61.6% (Figure 1a, b). Altogether, OLFM4 staining in cell cytoplasm was more common in HCC samples than in matched

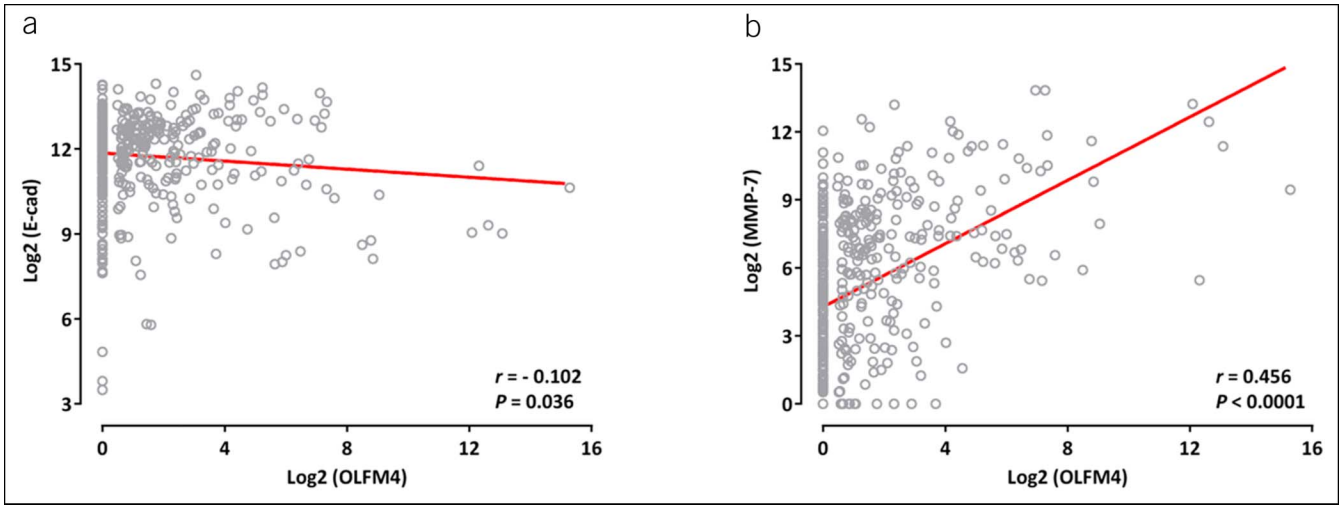

Figure 2. Assessment of the correlations between OLFM4 and E-cadherin (**a**) or MMP-7 (**b**) mRNA expression levels according to the analysis of an independent HCC cohort. HCC, hepatocellular carcinoma.

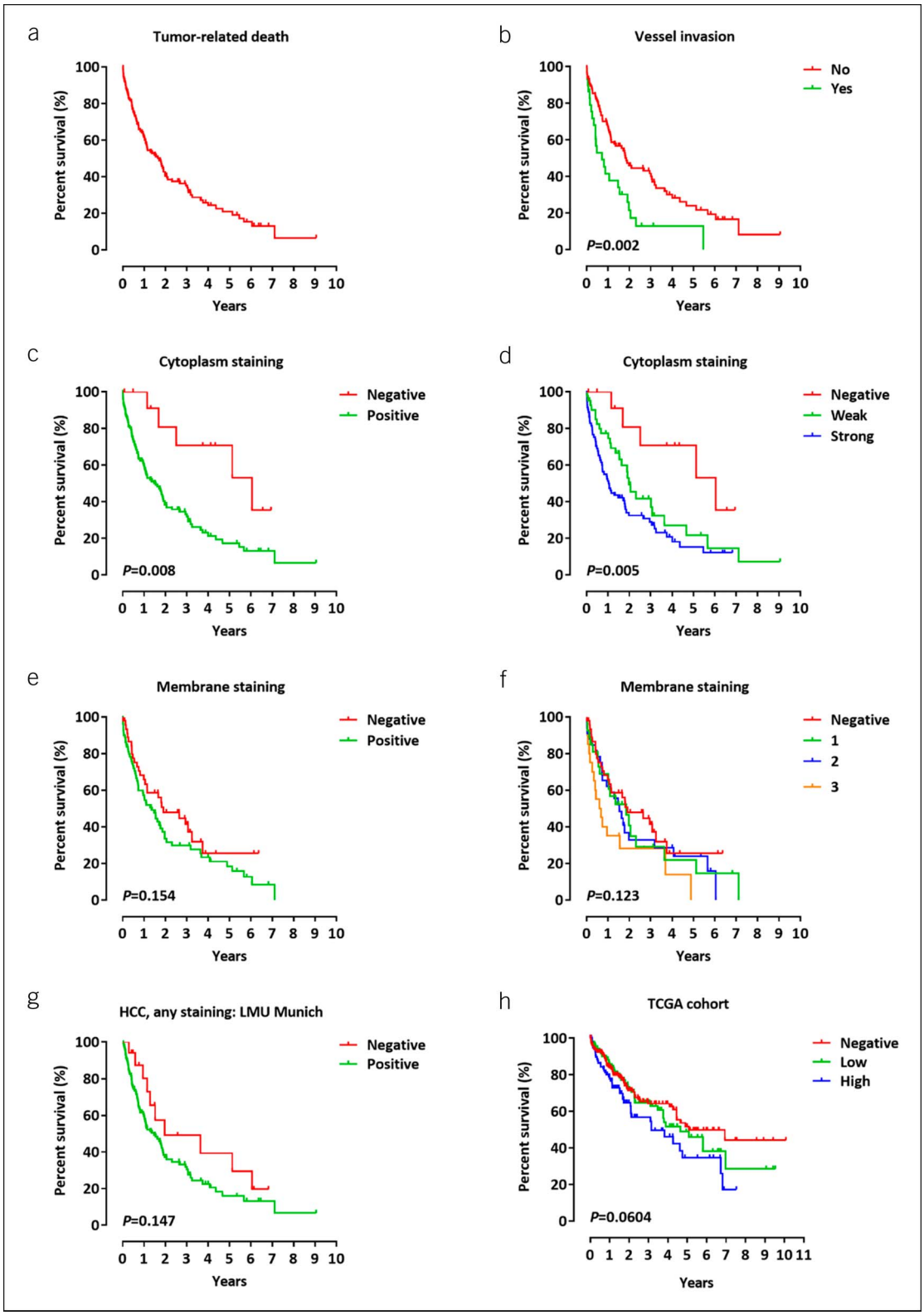

Figure 3. Prognostic significance of OLFM4 according to cellular distribution in patients undergoing hepatectomy. (**a**) Overall survival (OS) of the entire patients' collective as determined by the Kaplan-Meier method. (b) OS according to evidence of vessel invasion. OS according to cytoplasm staining for OLFM4 (positive or negative (**c**)) or according to semiquantitative assessment of staining intensity (**d**). OS according to membrane staining for OLFM4 (positive or negative (**e**)) or according to semiquantitative assessment of staining intensity (**f**). (**g**) OS according to OLFM4 staining in HCC tissues from LMU Munich. (**h**) Kaplan-Meier survival curves according to OLFM4 mRNA expression levels in a second independent cohort of patients with HCC from TCGA. +Censored cases. HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas.

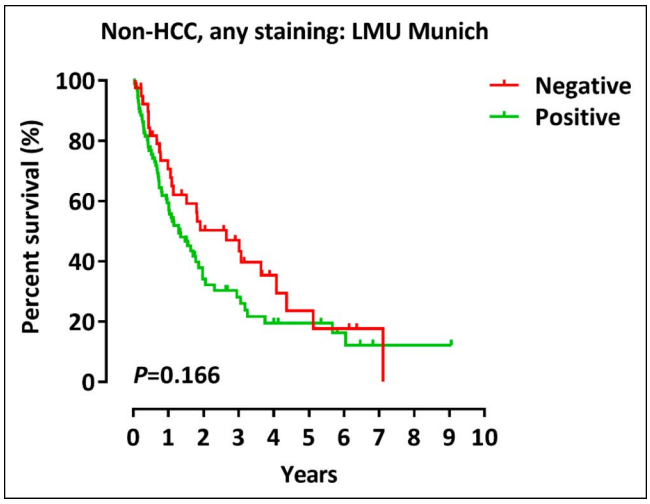

Figure 4. Overall survival according to OLFM4 staining in non-HCC tissues from LMU Munich cohort of hepatectomy. +Censored cases. HCC, hepatocellular carcinoma.

surrounding non-HCC lesions (OLFM4: 66.8% in normal cells vs 87.6% in HCC; Pearson's χ^2 test: $P < 0.001$). This difference was principally because of the higher fraction of HCC samples showing a pattern of mixed cytoplasmic and membrane staining for OLFM4 (61.6% of tumor samples vs 41.2% of nontumor samples exhibited positive membrane staining for OLFM4; Pearson's χ^2 test: $P < 0.001$).

Correlations between OLFM4 staining and clinicopathological characteristics of tumor tissues and nontumor tissue samples

We next assessed the correlation between OLFM4 staining in both tumor and nontumor specimens and the available demographic and clinicopathologic features of the collective, including etiology of underlying liver disease, age and gender of patients, presence of liver cirrhosis, grading and size of tumors, presence of vascular invasion, and treatment options. In addition, established immunohistochemical markers related to cell invasion and aggressive phenotype in HCC, including expression of E-cadherin (24) and MMP-7, were included in the analysis (Table 2).

Positive cytoplasmic staining for OLFM4 in tumor samples was associated with the presence of lobular inflammation in the surrounding nontumour tissue ($P < 0.001$), MMP-7 expression ($P = 0.002$), and blood vessel invasion ($P = 0.048$). Furthermore, a significant association was observed between membrane staining for OLFM4 and the expression of E-cadherin ($P = 0.042$) and MMP-7 ($P = 0.015$) in tumor samples. These results could be confirmed by analysis of mRNA expression data from the TCGA HCC cohorts, which showed a correlation between mRNA expression levels of OLFM4 and those of E-cadherin (Figure 2a, $r = -0.102$, $P = 0.036$) and MMP-7 (Figure 2b, $r = 0.456$, $P < 0.0001$), thus corroborating the finding that OLFM4 might contribute to worse prognosis by impinging on cell motility and invasion. In opposition to these correlations found in tumor samples, OLFM4 staining in matched nontumor tissues samples did not display any correlation with clinicopathological variables known to be associated with an aggressive phenotype (data not shown). Altogether, the high prevalence of OLFM4 staining in tumor tissue and the fact that its expression in tumor samples (but not in nontumor specimens) correlates with known markers of cell invasion corroborate the notion that OLFM4 plays a pro-carcinogenic role in HCC.

Cellular localization and prognostic significance of OLFM4 staining in tumor and nontumor samples

To assess whether the higher expression of OLFM4 could reflect a role of this molecule in determining an aggressive tumor phenotype, a survival analysis was performed according to the assessment of OLFM4 and other pathological variables in tumor specimens of patients undergoing partial hepatectomy (Figure 3a). A Kaplan-Meier analysis revealed, as expected, that the presence of vascular invasion (Figure 3b; $P = 0.002$) was a predictor of survival. In addition, however, patients' stratification according to OLFM4 staining (Figure 3c; $P = 0.008$) revealed that patients with positive OLFM4 staining in the cell cytoplasm had a poorer outcome compared with patients without cytoplasmic staining for OLFM4 (5-year overall survival: 58.3% vs 17.3%, respectively). This was confirmed by a survival analysis conducted by stratifying patients according to a semiquantitative assessment of OLFM4 cytoplasmic staining (for negative, weak, and strong staining intensity 5-year overall survival were 70.7%, 21.6%, and 15.7%, respectively, $P = 0.005$; Figure 3d). By contrast, survival was not affected by the presence of membrane staining (Figure 3e; $P = 0.154$, Figure 3f; $P = 0.123$).

The fact that cellular localization of OLFM4 rather than its overall staining determines the patients' survival was confirmed by the fact that survival analyses based on the presence or absence of OLFM4 staining, regardless of its cellular distribution or staining intensity, was not associated with the patients' outcome (Figure 3g, $P = 0.147$; Figure 4, $P = 0.166$). In addition, assessment of mRNA expression in the independent HCC cohort from the TCGA database showed that survival of patients exhibiting high OLFM4 expression was numerically lower, but not statistically different, than in patients with low mRNA expression (Figure 3h, $P = 0.0604$).

Taking into account different variables, including etiology, the presence of liver cirrhosis, multifocal lesions or vascular invasion, and the expression of E-cadherin, the Cox univariate analysis confirmed that cytoplasmic staining for OLFM4 (HR: 2.226; 95% CI: 1.186–4.177; $P = 0.013$) and vessel invasion (HR: 1.879; 95% CI: 1.181–2.989; $P = 0.008$) possess independent significance as determinant of survival of patients with HCC after partial hepatectomy (Table 3). In addition, multifocal HCC was associated with poorer prognosis in liver transplant recipients (Table 3, HR: 3.253; 95% CI: 1.407–7.52; $P = 0.006$; Figure 5, $P = 0.004$). The calculated HRs for OLFM4 cytoplasm staining was 2.135 (95% CI: 1.135–4.015; $P = 0.019$) and 1.791 for vessel invasion (95% CI: 1.124–2.854; $P = 0.014$; Table 4) in the multivariate analysis. By contrast, no correlation was found between OLFM4 staining in nontumor surrounding tissues and survival of patients (Figure 4, data not shown).

Silencing of OLFM4 suppresses cell migration and invasion and causes cell cycle arrest *in vitro*

To verify the hypothesis that OLFM4 determines an aggressive phenotype by influencing the metastatic properties of cancer cells and validate its association with MMP-7 and E-cadherin observed in tumor specimens, we assessed the effect of OLFM4 silencing by siRNA *in vitro*. As shown in Figure 6, effective silencing of OLFM4 could be achieved within 24 hours after transfection, an

Table 3. Cox regression of univariate analyses for survival in HCC tissues

	Hepatectomy			Transplantation		
Parameter	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
Age at diagnosis (y)						
<60						
≥60	0.946	0.624–1.434	0.795	0.715	0.313–1.634	0.426
Sex						
Male						
Female	1.084	0.669–1.755	0.743	1.049	0.398–2.766	0.923
Etiology						
HCV						
HBV	1.001	0.459–2.183	0.998	0.986	0.42–2.313	0.974
Toxic/metabolic	1.085	0.659–1.787	0.747	0.847	0.274–2.614	0.772
Severe fibrosis/cirrhosis						
<5						
≥5	1.286	0.844–1.958	0.242	0.951	0.414–2.184	0.905
Steatosis						
0						
1–3	0.725	0.473–1.111	0.140	0.563	0.256–1.24	0.154
Portal inflammation						
0–2						
3–4	1.202	0.787–1.835	0.395	1.176	0.57–2.428	0.661
Piecemeal necrosis						
0–2						
3–4	1.580	0.966–2.584	0.068	0.417	0.155–1.123	0.083
Lobular inflammation						
0–1						
2–3	0.702	0.434–1.133	0.148	0.866	0.328–2.283	0.771
Tumor size (cm)						
<5						
≥5	0.950	0.617–1.462	0.815	0.794	0.369–1.707	0.554
Extrahepatic metastasis						
No						
Yes	1.186	0.432–3.251	0.741	—	—	—
Grading						
1						
2	0.719	0.424–1.219	0.220	1.137	0.46–2.808	0.781
3	1.179	0.658–2.112	0.580	1.825	0.762–4.374	0.177
Blood vessel invasion						
No						
Yes	1.879	1.181–2.989	0.008[a]	0.643	0.267–1.55	0.325
Multifocal lesions						
No						
Yes	1.150	0.727–1.820	0.549	3.253	1.407–7.52	0.006[a]
E-cadherin						
0–1						

Table 3. (*continued*)

Parameter	Hepatectomy			Transplantation		
	HR	95% CI	*P*	HR	95% CI	*P*
2–3	0.623	0.342–1.137	0.123	1.14	0.336–3.874	0.833
MMP-7						
0–1						
2–3	1.028	0.590–1.790	0.923	1.165	0.471–2.881	0.74
OLFM4 cytoplasm staining						
Negative						
Positive	2.226	1.186–4.177	0.013[a]	0.821	0.331–2.038	0.671
OLFM4 membrane staining						
Negative						
Positive	1.216	0.781–1.891	0.386	0.983	0.466–2.075	0.964

CI, confidence interval; HCC, hepatocellular carcinoma; HR, hazard ratio.
[a]Statistical significance.

effect which was observable by both western blot analysis and immunofluorescence-based assessment of OLFM4 (Figure 6a, b). Downregulation of OLFM4 inhibited the expression of different members of the MMP family, comprising MMP-7, -2, and -9, but increased the expression of E-cadherin, which confirms the indirect association between OLFM4 with MMP-7 in immunohistochemical studies and its direct correlation to E-cadherin in tissue samples.

Because alterations of E-cadherin and MMP family molecules are involved in the process of cell invasion and the development of metastasis (25–27), we additionally performed scratch assays and transwell assays to determine the effect of OLFM4 silencing on cell motility and invasive properties *in vitro*. To this regard, we observed that cell motility was greatly diminished in OLFM4-siRNA transfected cells vs control cells as judged both by the assessment of healing areas at scratch assay (Figure 7a–c) and by decreased cell migration and invasion in transwell chambers (Figure 7d–h), showing that both along with loss of cell viability inhibition of cells motility, contribute to determine to decrease cell migration and invasive properties (27).

Finally, as OLFM4 was previously reported to affect apoptosis (28,29), we investigated whether OLFM4, besides affecting cell motility, also affects cell viability and proliferation. As shown by the significant increase of sub-G1 events after PI-staining, we found a small but significant increase of apoptosis after siRNA-OLFM4 incubation for 24 hours (Figure 7i, j). In addition, a G1 cell cycle arrests was observed, along with a corresponding decrease of the S and G2/M phases of cell cycle (Figure 7i, j).

Taken together, these results are in keep with the known role of E-cadherin and MMP in determining cell motility and invasion and are likely to represent the mechanism by which OLFM4 determines a poorer outcome in patients with HCC.

DISCUSSION

Staining, localization, and prognostic significance of OLFM4 in tumors vs surrounding nontumor tissues

OLFM4 is known as G-CSF-stimulated clone 1 protein (hGC-1) because it was originally cloned from human myeloblasts (30) in consequence of G-CSF administration, which reflects its immunomodulatory role and its effect on inflammation. In addition,

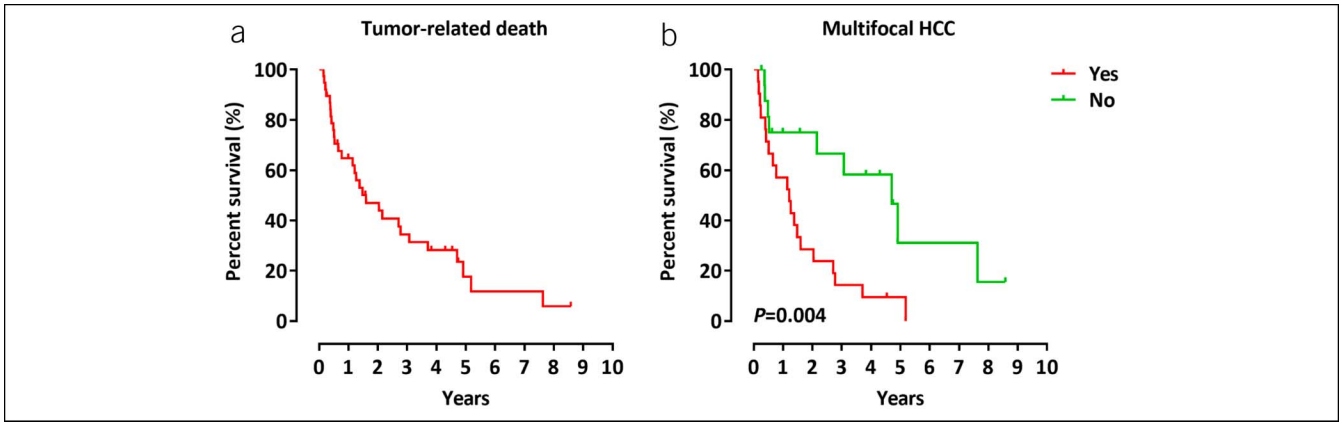

Figure 5. Overall survival (**a**) and prognostic significance of multifocal HCC (**b**) in liver transplant recipients from LMU Munich cohort. +Censored cases. HCC, hepatocellular carcinoma.

Table 4. Cox regression of multivariate analyses for OLFM4 staining in HCC tissues

Parameter	Hepatectomy		
	HR	95% CI	*P*
Blood vessel invasion			
No			
Yes	1.791	1.124–2.854	0.014[a]
Cytoplasm staining			
Negative			
Positive	2.135	1.135–4.015	0.019[a]

CI, confidence interval; HCC, hepatocellular carcinoma; HR, hazard ratio.
[a]Statistical significance.

OLFM4 is also implicated in a number of different function of cell physiology including regulation of cellular differentiation and cell death (5,31). Owing to this multiplicity of functions, it is not surprising that OLFM4 was shown to play a role in cancer development and possibly influence the response to chemotherapeutic treatment (13,15,32). Evidence provided on the specific role played by OLFM4 in cancer formation is, however, in part, contradictory because it was shown that OLFM4 may possess opposing effect in different contexts depending on the specific tumor entity and even on the stage of tumor development (5,15,33). However, to the best of our knowledge, no systematic study has been conducted to study the role of OLFM4 in HCC.

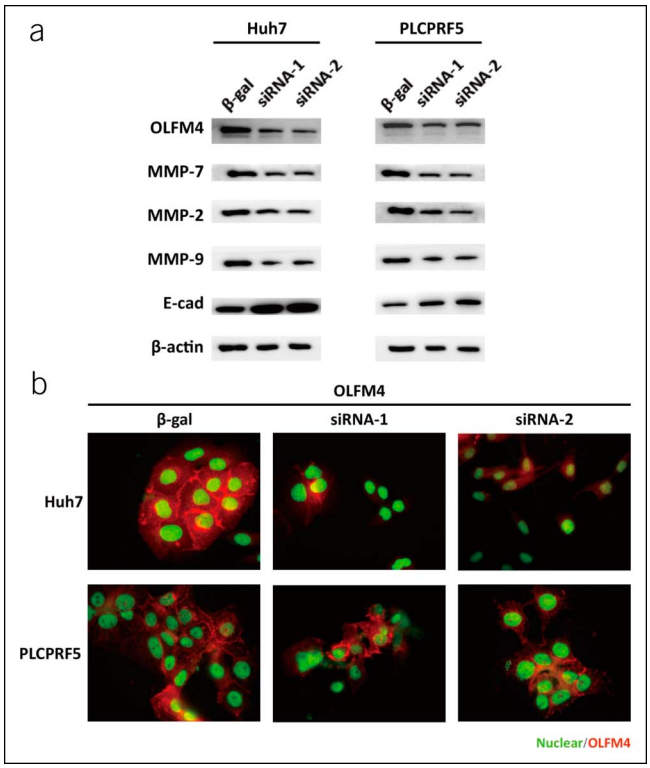

Figure 6. *In vitro* assessment of the effect of OLFM4 silencing on MMP-7 and E-cadherin. (**a**) Effect of transfection of Huh7 and PLCPRF5 cells with siRNA specifically targeting OLFM4 or non-coding siRNA (Beta-gal, 50 nM) for 24 hours on the expression of the indicated molecules after immunoblotting. (**b**) Typical pattern of OLFM4 expression at immunofluorescence after transfection with control-targeting or OLFM4-targeting siRNA. Green: DAPI staining of the nuclei; Red: immunofluorescence of OLFM4.

In agreement with the notion that OLFM4 plays a procarcinogenic role in cancer formation, we found that 87.6% of cancer samples vs 68.5% of matched nontumor samples exhibited a positive staining of OLFM4 (Figure 1). This is in accordance with previous findings showing that OLFM4 was significantly higher in serum of patients with solid tumors such as gastric, colorectal, pancreatic, head and neck, or prostate cancers than in healthy individuals (33). Interestingly, however, we did not find any association between patients' survival and overall staining for OLFM4. Similar results were obtained when we assessed the correlation between expression mRNA levels of OLFM4 and outcome of patients in an independent TCGA cohort. Instead, as tumor samples were categorized according to the cellular localization, we observed that tumor and nontumor samples essentially differed in the proportion of specimens with a cytoplasmic staining (Figure 1) and that five-year survival of patients with tumors exhibiting OLFM4 staining in cell cytoplasm had a poorer prognosis in comparison to patients without cytoplasmic staining for OLFM4 (Figure 3c, e and Tables 3 and 4).

Previous studies have reported that OLFM4 can be localized in virtually all cell compartments (10,17) and that may be also secreted (34) outside the cell and detected in plasma of patients. The predominant cytoplasmic localization of OLFM4 in tumor samples observed by us might represent the functional correlate of the interaction of OLFM4 with the cytoskeleton as previously described (15), and as suggested by the correlation with MMP-7 and E-cadherin observed by us, to modulate cell motility and the metastatic properties of cells.

Clinical-pathologic correlations of OLFM4 staining and functional assessment of OLFM4 *in vitro*

To assess the role of OLFM4 in the pathogenesis of HCC, we explored possible correlations between OLFM4 staining and pathological features defining the severity of the liver disease underlying the occurrence of HCC in the patients of our collective and hypothesized that increasing severity of liver disease (e.g. increasing grades of fibrosis, steatosis or lymphocyte infiltration) would be accompanied by increasing staining of OLFM4. To this regard, we observed that cytoplasm staining for OLFM4 in tumor samples had a significant association with lobular inflammation of the liver ($P < 0.001$). This association between OLFM4 and lymphocytic infiltration, which was previously described in specimens of patients affected by Crohn's disease or ulcerative colitis (33), might reflect an unfavorable effect of OLFM4 on the chronic inflammatory milieu, which represents the background of HCC formation (Table 2) (35).

In addition, as we assessed the correlation between OLFM4 staining and tumor-specific clinical-pathological characteristics, we found a correlation between cytoplasmic OLFM4, blood vessel invasion ($P = 0.048$), and MMP-7 expression ($P = 0.002$). Interestingly, increased staining for the E-cadherin was found only in association with the membrane-bound fraction of OLFM4 ($P = 0.042$; Table 2). This finding is of unclear significance, and it could be attributable to the fact that the adhesive activity of E-cadherin can be regulated at the cell surface by an inside-out

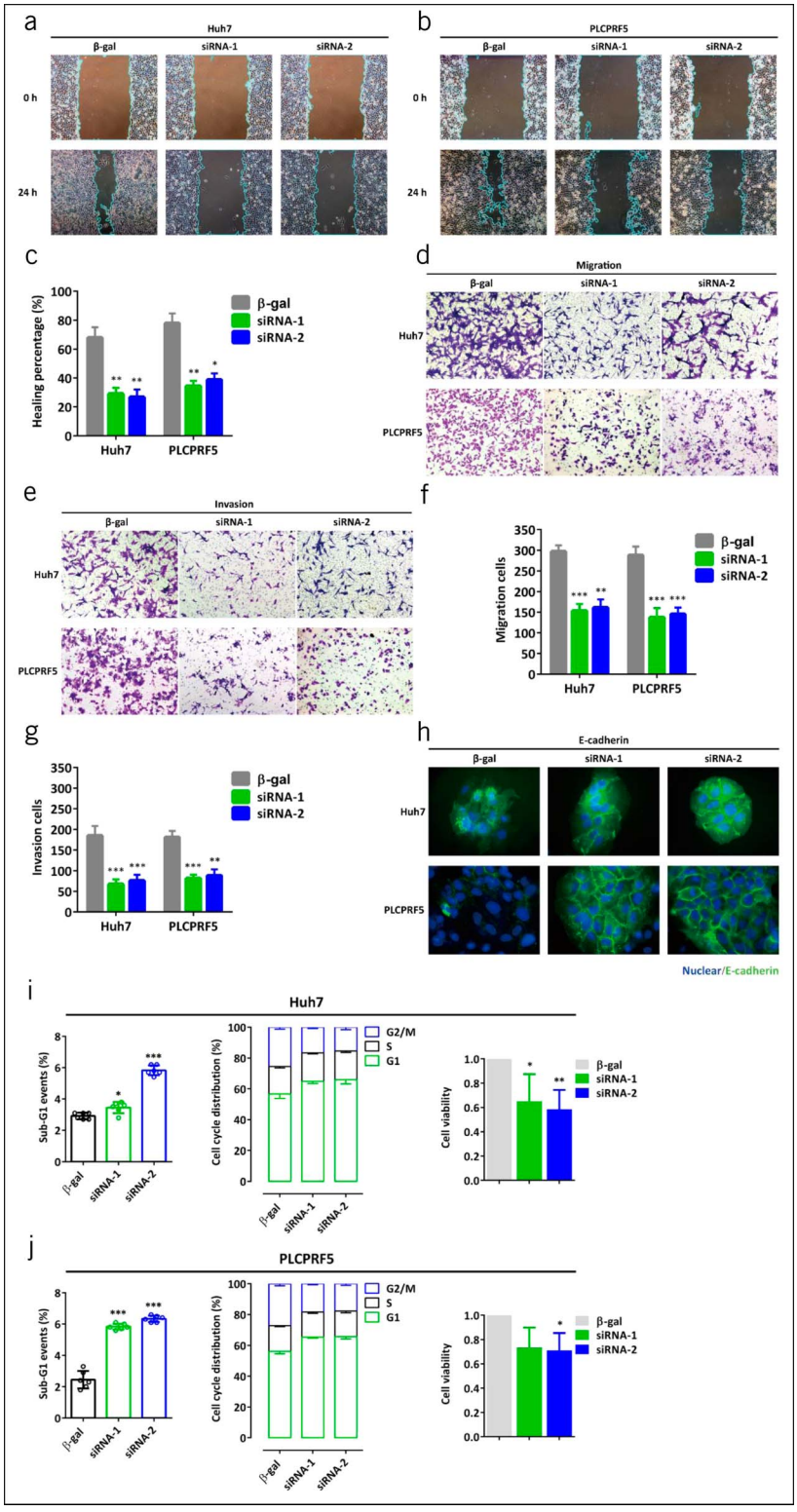

Figure 7. Effect of OLFM4 silencing on cell migration and invasion. (**a**, **b**) Assessment of cell motility by wound healing assay in Huh7 or PLCPRF5 cells transfected with siRNA-OLFM4 or control-si-RNA. (**c**) Quantitative analysis of healing area in percentages. (**d**, **e**) The migration and invasion of Huh7 and PLCPRF5 cells transfected with siRNA-OLFM4 compared with the controls by transwell assay after 24 hours. (**f**, **g**) Quantitative analysis of migration and invasion cells. (**h**) The E-cadherin expression of Huh7 and PLCPRF5 cells transfected with siRNA-OLFM4 compared with the controls by immunofluorescence after 24 hours. (**i**–**j**) Effects of OLFM4 silencing by siRNA on sub-G1 events, cell cycle distribution, and cell viability in Huh7 (**i**) and PLCPRF5 (**j**) cell lines. Values represented mean ± SD from 3 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Student *t*-test. Blue: DAPI staining of nuclei; green, immunofluorescence of E-cadherin.

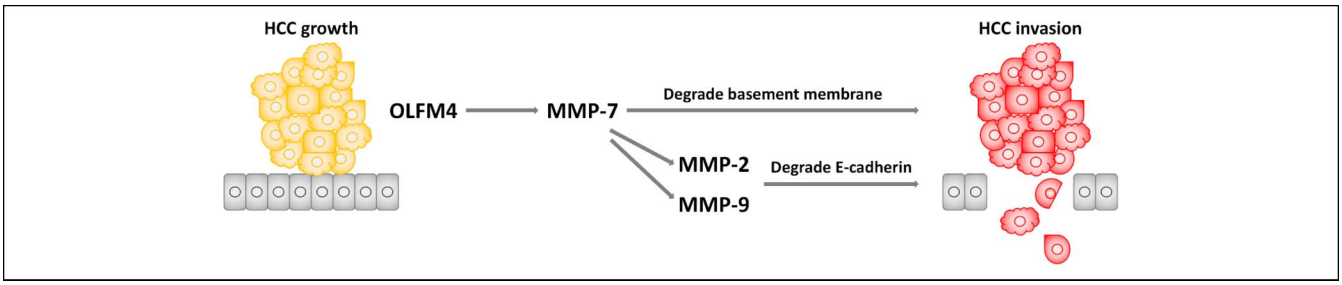

Figure 8. Schematic diagram describing the hypothesized effect of OLFM4, MMP family members and on invasion and metastasis formation in HCC. HCC, hepatocellular carcinoma.

signaling mechanism, as previously reported (36), and that other mechanisms other than the purported OLFM4-mediated degradation of E-Cadherin might play a role in determining cell invasion (37,38). However, a negative correlation between OLFM4 expression and E-cadherin and a positive one with MMP-7 could be confirmed by mRNA expression data (Figure 2) and by *in vitro* experiments, which showed that silencing of OLFM4 *in vitro* causes a decrease of MMP-7, MMP-2, and MMP-9 and a correlative increase of E-Cadherin. These *in vitro* experiments confirmed the functional role of OLFM4 in determining cell viability and metastatic properties of cancer cells. Collectively, our findings show that OLFM4 may play a role in regulating tumor invasiveness in HCC cells by activating MMP-7 expression, which causes degrading of the basal membrane and inhibits E-cadherin expression (Figure 8), thus contributing to the malignant phenotype of HCC.

Summary and possible clinical implications of OLFM4 expression in liver cancer cells

In summary, we provide the first report on the role of OLFM4 as a marker of aggressiveness in HCC. Our expression data in tumor vs nontumor tissue, the cellular localization of OLFM4, the confirmatory data from an independent cohort, and our *in vitro* assay collectively define OLFM4 as a regulator of cell adhesion and motility and a determinant of recurrence in early-stage HCC. In addition, our data suggest that OLFM4 might also affect cell proliferation and apoptosis (Figure 7i, j) and contribute to determine the inflammatory microenvironment, which is known to underlie the formation of most HCC cases (Table 2).

We thus propose that OLFM4 is studied as a biomarker with the potential of identifying patients at risk of recurrence or, as previously suggested (5,33), response to treatment. To this regard, it was shown that serum levels of OLFM4 could be used as a circulating biomarker for gastric and colorectal tumors (13,15,39). Whether this could be translated to the case of HCC—thus providing a circulating marker of aggressiveness and possibly of response to immune checkpoint inhibitors (40)—will be the interesting object of future studies.

Our study also highlights the importance of MMP/E-Cadherin signaling in determining the prognosis of early-stage tumors. Future studies will have to assess the role played by OLFM4 within well-established, potentially actionable signaling pathways, such as the PI3K-Akt-Tor signaling, beta-catenin, RAS-RAF-MEK-ERK, PDGF, and MET signaling. In addition, small molecule screening investigations should be conducted to detect substances capable of modulating this pathway in the specific context of adjuvant treatment.

CONFLICTS OF INTEREST

Guarantor of the article: Enrico N. De Toni, MD.

Specific author contributions: L.T.Y. and L.K. performed the research. L.T.Y. collected and analyzed the data. L.T.Y. and E.N.D.T. designed the research study and wrote the manuscript. F.P.R., S.M.M., T.I., A.T., A.Z., and H.P.T. contributed to the design of the study. T.K., A.L.G., M.G., and J.M. reviewed the manuscript.

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Potential competing interests: All authors approved the final version of the manuscript and declared no conflicts of interest.

Study Highlights

WHAT IS KNOWN

✓ Recurrence of HCC is common even in patients treated with curative intent. No biomarker-driven or stage-specific systemic treatment is available.

WHAT IS NEW HERE

✓ OLFM4 overexpression represents an independent marker of aggressive phenotype in early-stage HCC. At functional level, we define the role of OLFM4 as a regulator of MMP-7 and E-cadherin in determining HCC cell invasion and metastasis.

TRANSLATIONAL IMPACT

✓ OLFM4 may be used as a biomarker to identify patients at risk of HCC recurrence and to establish novel adjuvant treatment options for HCC.

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