De novo synthesis of C4.4A in hepatocellular carcinoma promotes migration and invasion of tumor cells

MAGDALENA GÖRTZ[1], UWE GALLI[1], THOMAS LONGERICH[2], MARGOT ZÖLLER[3], ULRIKE ERB[3] and PETER SCHEMMER[1,4]

[1]Department of General and Transplant Surgery, [2]Institute of Pathology, [3]Tumor Cell Biology, Department of General and Transplant Surgery, University Hospital Heidelberg, Heidelberg, Germany; [4]Department of Surgery, Division of Transplant Surgery, Medical University of Graz, Graz, Austria

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Abstract. C4.4A is a glycoprotein that is upregulated in several human malignancies, including colorectal, breast and renal cell carcinomas. Due to its highly restricted expression in healthy tissue, C4.4A was proposed as a potential diagnostic marker. Thus, the present study was designed to evaluate C4.4A expression and function in hepatocellular carcinoma (HCC) for the first time. Immunohistochemistry was performed to detect expression of C4.4A in human sections of healthy liver, primary HCC in the liver and metastatic HCC in the lung. To assess the contribution of C4.4A to HCC progression proliferation, apoptosis, migration and invasion assays were performed with C4.4A knockdown Huh7 and HepG2 cells. C4.4A is absent in healthy liver tissue. However, intense expression was seen in 59% of primary HCCs and strong expression in 80% of HCC lung metastases. C4.4A expression was also observed in human HCC cell lines, which strongly increased under hypoxic conditions. A C4.4A knock-down revealed that C4.4A is involved in both migration and invasion of HCC cells. Taken together, C4.4A expression in both primary and metastatic HCC suggests its potential value as a diagnostic marker for HCC. Due to its absence in healthy liver tissue, C4.4A might even serve as a possible therapeutic target, particularly for metastatic HCC.

Introduction

Hepatocellular carcinoma (HCC), a primary malignancy of the liver, has become over the past years the sixth most common cancer worldwide (1,2). The HCC is characterized by rapid cell growth, early intrahepatic metastasis, high grade malignancy and drug resistance, which result in poor 5-year survival rates of only 5% (3,4). The best long-term survival in patients with HCC is achieved by liver resection (5) or liver transplantation (6,7); however, there is no indication for resection of tumors that have already spread to regional lymph nodes or distant organs (8-10). Effective treatment of HCC remains a challenge because early diagnostic markers and effective therapeutic options are still pending, and the molecular mechanisms that contribute to carcinogenesis and progression of HCC have not yet been fully clarified. HCCs are discovered at a late stage of disease in most cases and thus the diagnosis is given at a time-point when therapeutic options are limited. Diagnostic markers for HCC would be of upmost importance for the diagnosis of an early tumor stage, which allows for curative treatment (11,12). Additionally, due to the high incidence of both recurrent and metastatic HCC, studies on the mechanisms of tumor invasion are desperately needed (13,14).

C4.4A was found to be highly expressed in several types of carcinomas such as non-small cell lung, colorectal and renal cell cancers (15-17). In the majority of studies, high C4.4A expression levels in the primary tumor correlated with poor patient survival (18,19). Although its exact function is still unknown, C4.4A is frequently detected in tumor metastases and in wound healing which is pointing toward a role in cell migration (20,21). This hypothesis was confirmed in several studies demonstrating the engagement of C4.4A-expressing tumor cells in migration and invasion (20,22). Notably, a recent study with C4.4A-deficient bladder carcinoma in C4.4A-knockout mice demonstrated unimpaired primary tumor growth, but decreased invasion capability (23).

Most interestingly C4.4A is rarely expressed in healthy organs, including the liver (17,24). This suggests C4.4A as a potential diagnostic marker. Next to the option of a diagnosis with scintigraphy, C4.4A may also be detected in serum exosomes, as C4.4A is readily recovered in exosomes in metastatic pancreatic carcinoma (21).

In addition, C4.4A might serve as a therapeutic target. First preclinical results of a phase I study (NCT02134197) with a C4.4A-directed antibody-drug conjugate (BAY1129980) in non-small cell lung cancer showed a sufficient antitumor efficacy in *in vivo* models (25).

Correspondence to: Professor Peter Schemmer, Department of Surgery, Division of Transplant Surgery, Medical University of Graz, Auenbruggerplatz 29, 8036 Graz, Austria
E-mail: peter.schemmer@medunigraz.at

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C4.4A expression has not yet been evaluated in HCC. Thus, the present study was designed to explore whether C4.4A could serve as a potential diagnostic marker or therapeutic target in patients with HCC.

Materials and methods

Human tissue and cell lines. Human tissue samples from non-inflammatory and non-tumorous livers, colorectal liver metastases and hepatocellular carcinoma (HCC) were collected during surgery. Tissue from lung metastasis of HCC was kindly provided by the Tissue Bank of NCT Heidelberg. The samples were used in accordance with the rules of the Tissue Bank and approved by the ethics committee of the Heidelberg University. Both human HCC cell lines, Huh7 (European Collection of Cell Cultures) and HepG2 (Toni Lindl GmbH, Munich, Germany), were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin in 5% CO_2 at 37˚C. Cells grew adherently and, when confluent, were detached with trypsin for sub-culturing. A Modular Incubator Chamber (Billups-Rothenberg, Inc., San Diego, CA, USA) was used to culture the cells under hypoxic conditions.

Antibodies. The following prevalidated antibodies were used: C4.4A (rabbit anti-human, IBL; sheep anti-human, R&D Systems, Minneapolis, MN, USA), actin (mouse anti-human; Sigma-Aldrich, St. Louis, MO, USA), donkey anti-sheep HRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-mouse HRP (Santa Cruz Biotechnology), Cy3-conjugated donkey anti-sheep IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA), goat anti-rabbit APC (Jackson Immunoresearch Laboratories), APC-Annexin V (Becton-Dickinson, San Diego, CA, USA) and PI (Becton-Dickinson).

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissue sections (5 μm) were deparaffinised by three 5-min washes in Roticlear and rehydrated using a series of ethanol/water solutions. Antigen retrieval was achieved by boiling in 1X citrate-based, unmasking fluid (pH 6.0) for 2x7 min in a microwave oven. Sections were treated with 3% hydrogen peroxidase/phosphate-buffered saline (PBS) for 5 min, followed by two washes in PBS. Immunohistochemistry was carried out using the Sheep IgG Vectastain ABC-AP kit (Vector Laboratories, Inc., Burlingame, CA, USA). The slides were incubated overnight at 4˚C with the primary human C4.4A antibody at a concentration of 1 μg/ml. After three washes in PBS, the tissue sections were incubated with biotin-conjugated, secondary antibody (rabbit anti-sheep IgG) and Vectastain ABC-AP reagent. The sections were developed using the Vector Red Alkaline Phosphatase substrate kit for 30 min as specified by the manufacturer. Finally, the sections were counterstained with Mayer's hematoxylin for 10 sec, dehydrated in ethanol and then mounted. Immunohistochemistry was examined using a Zeiss Axiovert 40 CFL microscope.

Western blot analysis. Huh7 cells and HepG2 cells were lysed with RIPA buffer. The suspension was put on ice for 10 min and then scraped with a plastic cell scraper prior to ultrasonification. Finally, the lysate was cleared by centrifugation at 4˚C, 10.000 x g. Protein lysates (7.5 μl) were separated on a 4-12% SDS-PAGE Bis-Tris gel, transferred to a nitrocellulose membrane (Bio-Rad Laboratories GmbH, München, Germany) and blocked in 5% non-fat dried milk. After incubating overnight with the primary antibody, the protein was visualized with the appropriate horseradish peroxidase-coupled, secondary antibodies (Santa Cruz Biotechnology) using SuperSignal West Pico Chemiluminescent substrate (Thermo Fisher Scientific, Rockford, IL, USA).

Immunofluorescence. Cells seeded on coverslides were fixed with ice-cold methanol. After blocking, the cells were incubated at 37˚C for 45 min with primary antibody. Antibody diluent with background reducing components from Dako (Glostrup, Denmark) was used to dilute the primary antibodies and as a negative control. After five washes in PBS, the cells were incubated with fluorochrome-conjugated, secondary antibody at 37˚C for 45 min before being washed again with PBS. Nuclear staining was achieved with DAPI (Sigma-Aldrich). Finally, coverslides were mounted in a fluorescence mounting medium from Dako.

Flow cytometry. Cells ($2x10^5$) were fixed and permeabilized for intracellular staining. After incubation with 30 μl primary antibody (19) at a concentration of 2.5 μg/ml (30 min, 4˚C), cells were washed two times and incubated with dye-labeled secondary antibody (30 min, 4˚C). Finally, cells were analyzed in a FACSCalibur using the CellQuest analysis program (BD Biosciences, Heidelberg, Germany).

siRNA transfection. For a C4.4A knockdown in human HCC cell lines, the following predesigned and prevalidated siRNAs were purchased from Qiagen: siC4.4A (cat. no. SI00105707) and siControl (cat. no. SI03650318). The Huh7 and the HepG2 cell lines were transfected with siRNA using HiPerFect Transfection reagent (Qiagen, Hilden, Germany) according to the supplier's protocol. Transfection efficiency was evaluated after 48 h by flow cytometry.

Cell proliferation assay. Cell proliferation was analyzed by the MTT assay. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well at 24, 48 and 72 h after siRNA transfection. The plates were incubated for 4 h before addition of propanol. The absorbance was measured in 96-well plates at 570 nm using a microplate reader.

Cell apoptosis assay. Cells were treated with 2 μg/ml of cisplatin to induce apoptosis. At 48 h after siRNA transfection cells were harvested and resuspended in binding buffer. After addition of APC-Annexin V and propidium iodide (PI) the cells were incubated in the dark for 15 min at room temperature and then analyzed by flow cytometry (FACSCalibur; BD Biosciences).

Migration and invasion assays. Migration and invasion of HCC cells were evaluated in Boyden chambers using a cell migration assay and a laminin-coated cell invasion assay (Cell Biolabs, San Diego, CA, USA). Twenty-four hours after siRNA

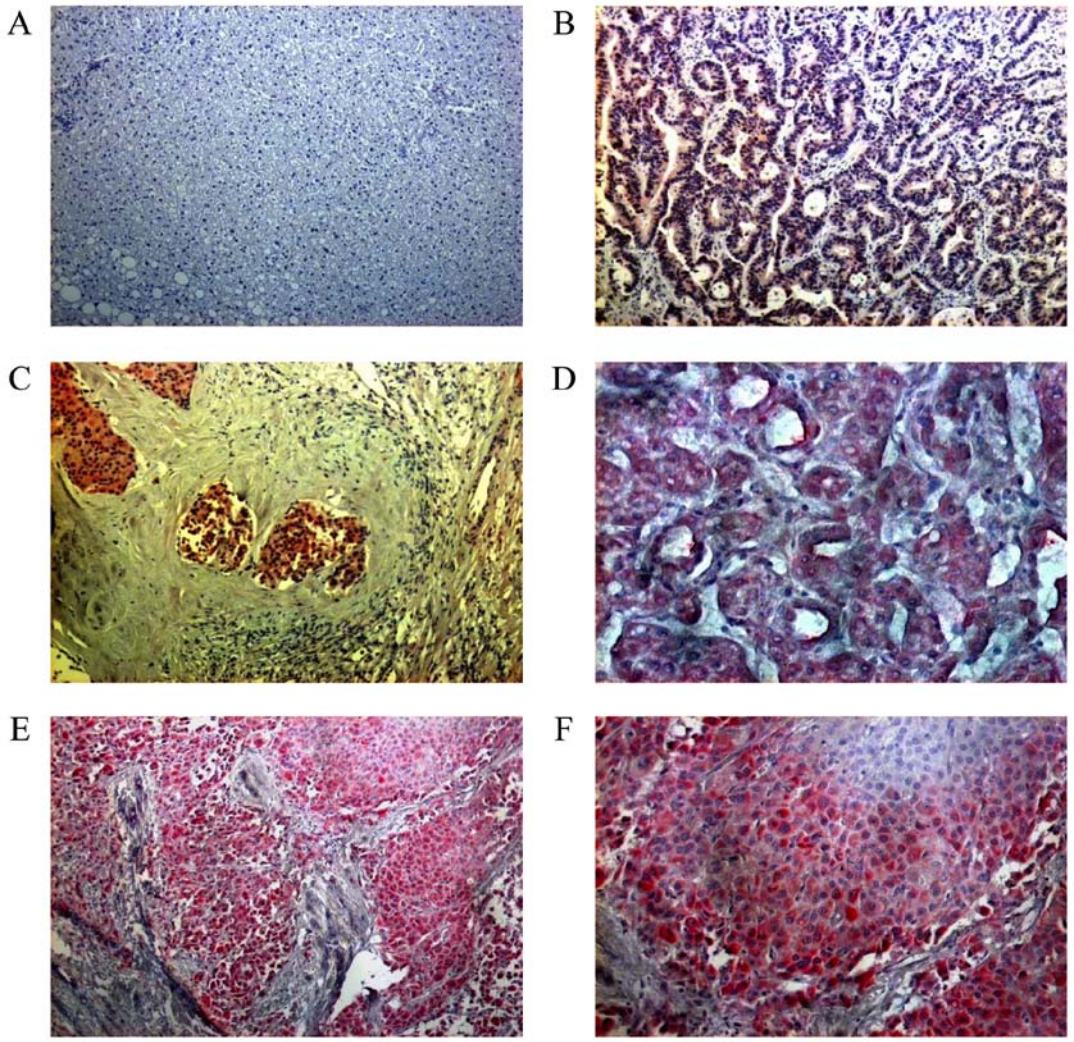

Figure 1. Expression of C4.4A in liver tissue. (A-F) Immunohistochemical analysis of C4.4A expression in human liver tissues. (A) Healthy liver tissue; (B) liver metastasis of colorectal cancer; (C) HCC; (D) HCC with pseudoglandular growth pattern; (E and F) pulmonary HCC metastases. Magnification of A-C and E, x100; D, x40x; F, x200.

transfection, cells were seeded in serum-free medium in the upper chamber. The lower chamber, which was separated by an 8 μm pore size polycarbonate-membrane, contained medium supplemented with 10% FCS. After incubation for 12-48 h at 37˚C, the non-migratory cells on the upper chamber were removed by a cotton swab and the cells that migrated to the undersurface of the membrane were stained with crystal-violet. The number of migrated cells was counted within a field at x100 under a light microscope. For each membrane, a total of five fields were selected at random and the numbers were averaged.

Statistical analysis. The values obtained are the mean (±SEM) of three replicates. Statistical analysis was performed using the unpaired, two-tailed t-test. Significance was established at a value of $P<0.05$.

Results

C4.4A-expression in human liver tissue. C4.4A-expression was evaluated in 12 samples of non-inflammatory and non-tumorous liver, 17 HCCs and 10 pulmonary HCC metastases. C4.4A-expression was not detected in any of the 12 samples of non-inflammatory and non-tumorous liver tissue (Fig. 1A). Since C4.4A was reported to be expressed in liver metastasis from colorectal carcinoma (17), samples from metastatic colorectal carcinoma (Fig. 1B) were used as C4.4A-positive reference tissue.

Distinct C4.4A-expression was seen in 10 out of 17 (59%) HCC samples (Fig. 1C). In addition to the presence of cyto-plasmic staining, there was increased membranous C4.4A expression on the luminal side of the glandular structures in HCCs with a pseudoglandular growth pattern (Fig. 1D).

C4.4A is absent from healthy bronchial and alveolar tissue (26); however, it was found to be highly expressed in samples of pulmonary HCC metastases (Fig. 1E). Eight out of 10 (80%) samples were C4.4A-positive. The strongest C4.4A-expression was noted at the invasive front of the liver tumor nodules, namely in hepatocytes located near the fibrotic septa and next to the surrounding lung tissue (Fig. 1F).

In summary, C4.4A was determined to be absent from non-inflammatory and non-tumorous liver, upregulated in primary HCCs (59%) and strongly expressed in 80% of HCC metastases.

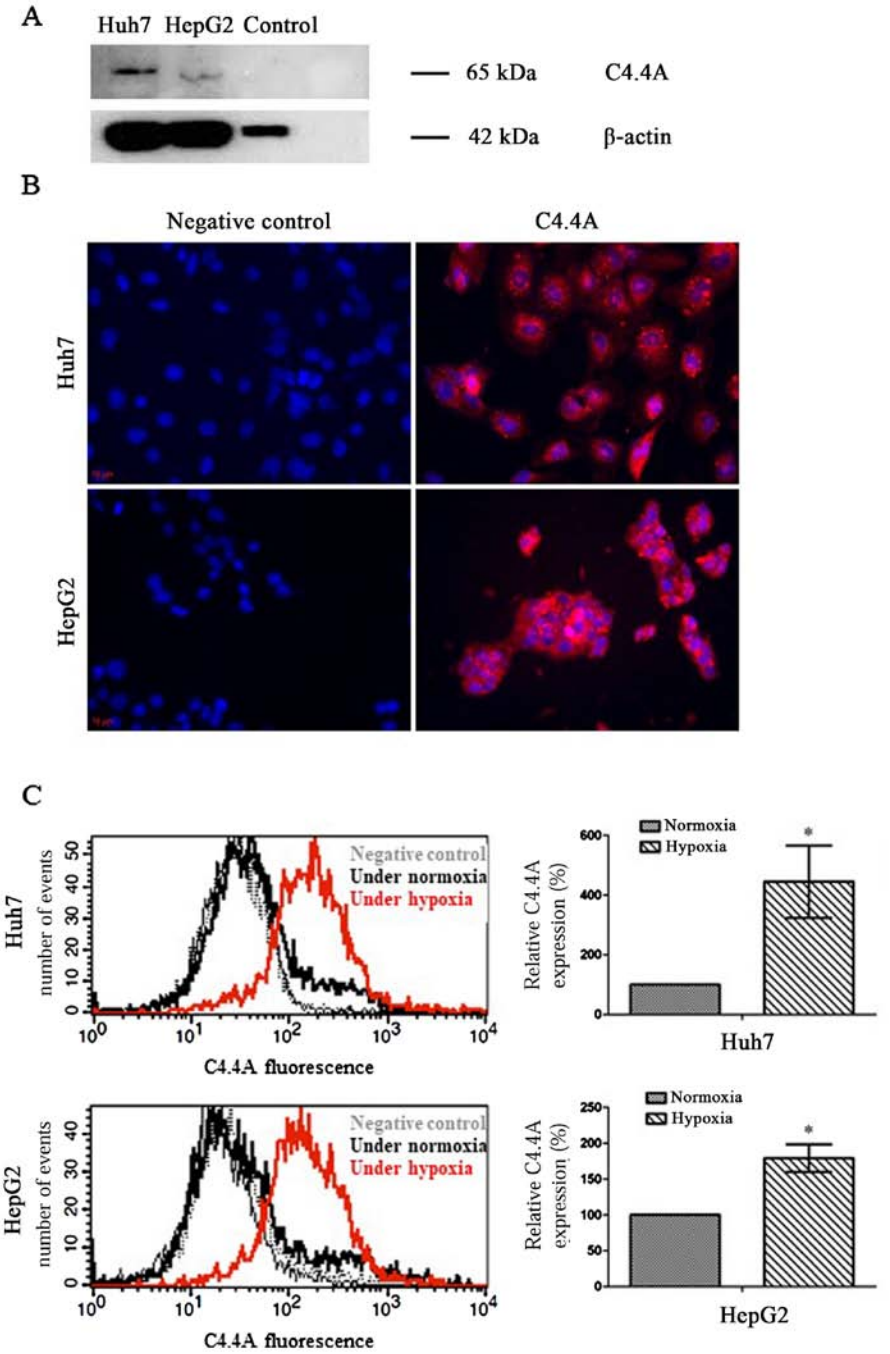

Figure 2. C4.4A expression in HCC cells under normoxia and hypoxia. (A) Human HCC cell lines Huh7 and HepG2 showed a specific signal for C4.4A at 65 kDa, whereas normal liver tissue (negative controls) did not show C4.4A expression. (B) The nuclei of HCC cells were stained with DAPI (blue fluorescence), the C4.4A antibody was detected by a Cy3-conjugated secondary antibody (red fluorescence). Negative controls without primary antibody were added for both cell lines. Magnification, x400. (C) C4.4A expression increased after incubation under hypoxia for 6 h compared to normoxia. Negative controls without primary antibody were added for both groups. The results from representative experiments are shown on the left panel and the calculated numbers from triplicates are shown on the right panel. *P<0.05.

Upregulation of C4.4A in HCC cell lines under hypoxic conditions. Firstly, it was determined whether or not C4.4A was present in HCC cell lines. Western blot analysis revealed a specific signal for C4.4A in both Huh7 and HepG2 human liver cancer cells with a monomer band of ~65 kDa (Fig. 2A). As previously determined (17), normal liver tissue did not express C4.4A and as such, could be used as negative controls.

As shown in Fig. 2B, immunofluorescence revealed that C4.4A was localized mainly within the cytoplasmic compartment of both human cell lines. C4.4A expression was significantly increased under hypoxic compared to normoxic culture conditions (Fig. 2C).

Downregulation of C4.4A does not influence HCC cell proliferation or apoptosis. C4.4A was knocked down in both Huh7 and HepG2 cells by siRNA to examine the impact of C4.4A on HCC tumor progression. C4.4A expression was strongly reduced in Huh7 and less pronounced, though significant in HepG2 cells at 48 h after siC4.4A transfection (P<0.05) (Fig. 3A).

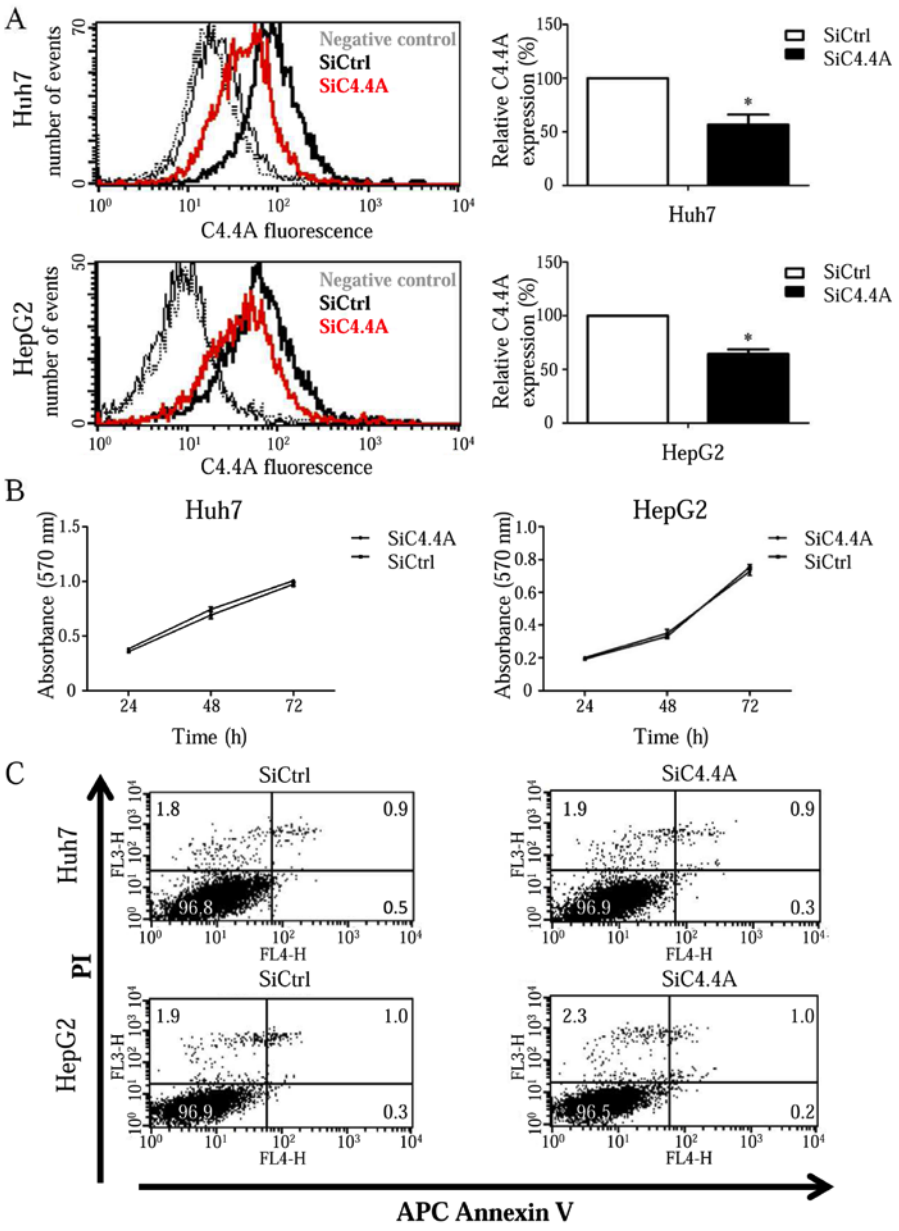

Figure 3. Influence of C4.4A on HCC cell proliferation and apoptosis. (A) The transfection efficiency was determined 48 h after transfecting Huh7 and HepG2 cells with C4.4A-siRNA or negative control siRNA. Negative controls without primary antibody were added for both groups. The results from representative experiments are shown on the left panel and the calculated numbers from triplicates are shown on the right panel. *P<0.05. (B) Cell proliferation activity after transfection of Huh7 and HepG2 cells with C4.4A-siRNA or negative control siRNA was assessed by MTT assay. The curves of cell growth after transfection for 24, 48 and 72 h are shown. Results are given as means ± SEM from three independent experiments. (C) Forty-eight hours after transfection with C4.4A-siRNA or negative control siRNA, cell apoptosis was determined by flow cytometry using Annexin V and PI labeling. Experiments were performed three times and representative analyses are shown.

Proliferation of both Huh7 and HepG2 cells was evaluated by the MTT assay at 24, 48 and 72 h after transfection. The number of viable cells increased overtime and there was no evidence of differences between siC4.4A-treated cells and controls (Fig. 3B).

Downregulation of C4.4A did not affect the viability of either Huh7 or HepG2 cells as demonstrated by Annexin V and PI staining in samples taken at 48 h after transfection. Both the control and the C4.4A knockdown Huh7 cells showed ~0.4% Annexin V-positive, ~0.9% Annexin V- and PI-positive and ~1.9% necrotic (PI-positive) cells. Similar results were obtained for the control and C4.4A knockdown HepG2 cells [~0.3% Annexin V-positive, ~1.0% Annexin V- and PI-positive and ~2.1% necrotic (PI-positive) cells] (Fig. 3C).

Downregulation of C4.4A reduces HCC cell migration and invasion. It has been reported that C4.4A is involved in the migration and invasion of colorectal and head and neck carcinoma (20,27). Whether this also accounts for HCC cells was evaluated in Huh7 and HepG2 cells after downregulation of C4.4A by siRNA treatment. Transwell migration was evaluated in a Boyden chamber assay using membranes with a 0.8 μm pore size. After incubation for 12 h (Huh7) or 24 h (HepG2), the lower membrane site was stained and cells in 5 fields were counted. Downregulation of C4.4A significantly

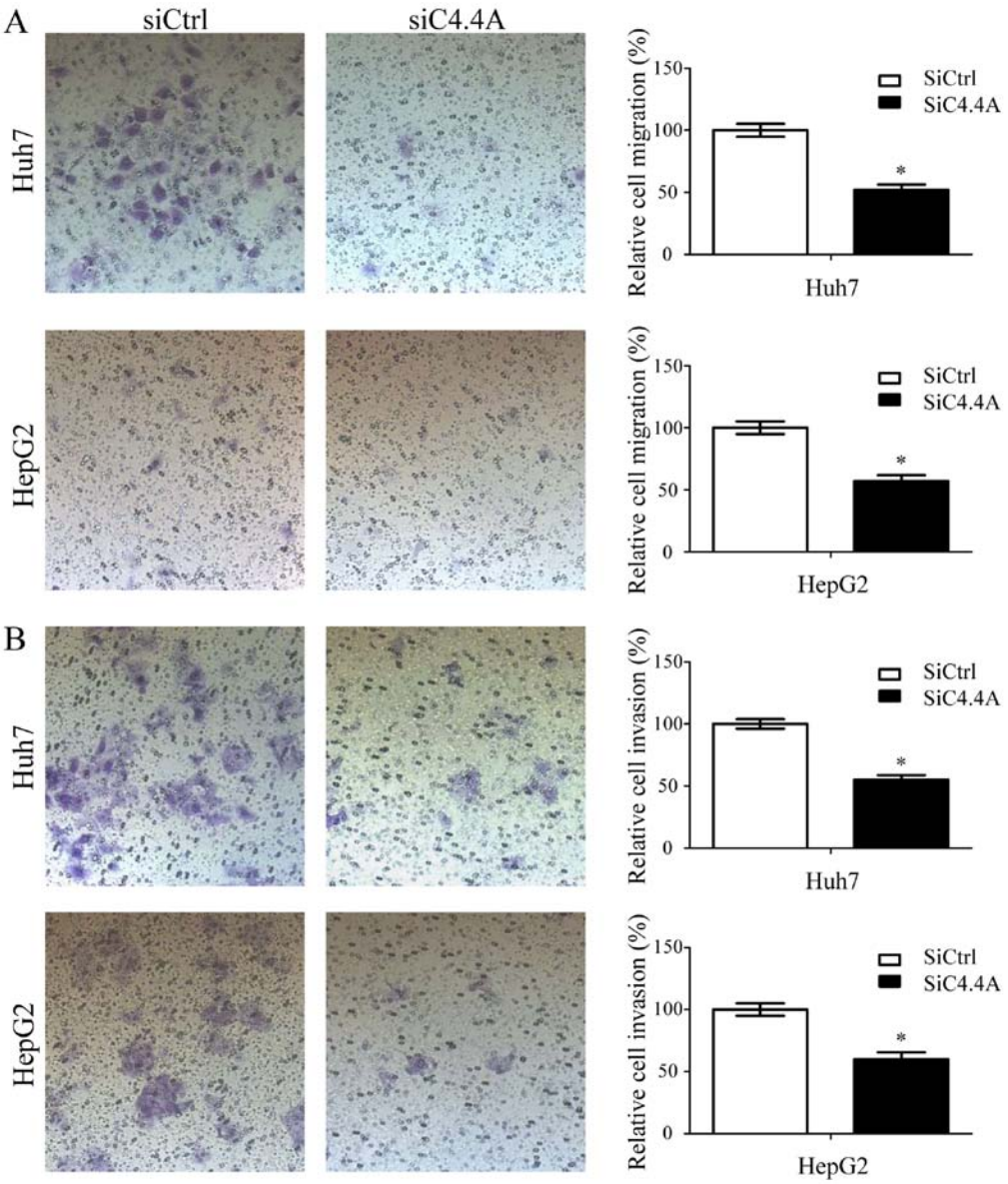

Figure 4. Influence of C4.4A on HCC cell migration and invasion. Downregulation of C4.4A reduces HCC cell migration (A) and invasion (B). Huh7 and HepG2 cells were either transiently transfected with unspecific negative control siRNA (siCtrl) or with C4.4A-specific siRNA (siC4.4A). The results from representative cell migration and invasion assays are shown on the left panel and the calculated numbers from triplicates are shown on the right panel. *P<0.05.

decreased the ability of both Huh7 and HepG2 cells to migrate through the membrane (Huh7, 111±10 vs. 57±8; HepG2, 40±4 vs. 23±3 cells per field; P<0.05; Fig. 4A).

In a Transwell invasion assay, downregulation of C4.4A significantly decreased the number of both Huh7 and HepG2 cells that penetrated through Matrigel (Huh7, 202±14 vs. 112±12; HepG2, 102±9 vs. 62±10 cells per field; P<0.05) (Fig. 4B). These results indicate that the migratory and invasive potential of HCC cells is significantly reduced by a C4.4A knockdown.

Discussion

This is the first report on C4.4A expression in liver. While in the healthy liver hepatocytes are C4.4A negative, C4.4A expression is strongly expressed in HCC with upregulation at the invasive front and in lung metastasis, indicating that C4.4A apparently contributes to HCC progression. We will discuss these findings in view of known features of C4.4A.

C4.4A was first described as a marker of a metastasizing rat pancreatic adenocarcinoma (22,28). Distinct to several other metastasis markers, C4.4A is rarely expressed in non-transformed cells. Expression of the human C4.4A was observed in placental tissue, skin, esophagus and very weakly in subpopulations of blood leukocytes, but not in brain, lung, liver, kidney, stomach, colon and lymphoid organs (29). C4.4A expression in the skin epithelium becomes upregulated during wound healing (30). Consequently, it was suggested that C4.4A is engaged in migration and/or invasion. This has been confirmed *in vivo* for wounded skin epithelia as well as for metastasizing tumor cells (21,27,31). *In vitro* assays confirmed pronounced migration and invasion, where it also was demonstrated that invasion relies on cooperativity of C4.4A with proteases. Our studies confirm these findings with strongly

reduced migratory and invasive capacity of C4.4A-knockdown HCC lines.

It also should be mentioned that using the C4.4A-knockdown HCC lines there was no evidence for an engagement of C4.4A in proliferative activity or apoptosis resistance, two features frequently associated with metastasis-prone tumor cells. Similar findings were reported for colon cancer cells (20), but for pancreatic cancer cells an engagement in apoptosis induction and proliferation was reported (32). A most likely explanation could be that C4.4A expression is not sufficient to fulfill these tasks, but C4.4A may act as a coactivator. It then depends on different expression profiles of distinct tumor entities, whether or not C4.4A supports apoptosis resistance and proliferation. Further studies are needed to address this question.

One of the notable findings in this study has been the extremely strong upregulation of C4.4A in HCC lines maintained under hypoxic conditions. This is important as hypoxic conditions are common in HCC and often result in enhanced tumor progression and metastasis (33-35). Promoter studies revealed so far a contribution of C/EBP beta and JunD or c-Jun, but no contribution of HIF-1α for C4.4A transcription (36). Further promoter analyses may unravel the cotranscription factors accounting for the highly increased expression under hypoxic conditions. Concerning C4.4A as a potential diagnostic marker, the strong upregulation under hypoxia, which is frequently seen in HCC, adds to its diagnostic potential.

Finally, first studies with a C4.4A knockout mouse need to be mentioned as they confirmed again the engagement of C4.4A in the metastatic spread of tumor cells without evidence for functional activity as an oncogene.

In brief, the C4.4A protein is absent from normal liver, but strongly expressed in HCC and its metastasis. C4.4A expression in HCC cells is further enhanced by hypoxic conditions. In Huh7 and HepG2 cells, C4.4A is engaged in tumor cell migration and invasion.

Conclusion and outlook. Metastasis associated molecules are in many instances abundantly expressed. C4.4A is exceptional with displaying very restricted expression in non-transformed tissue. This also accounts for the liver, where it is highly expressed in 59% of primary HCC and in 80% of lung metastasis.

As the prognosis of patients with HCC is poor and reliable diagnostic markers are limited, C4.4A might well serve as a new diagnostic and/or prognostic marker. HCC develops in cirrhotic livers, where differential diagnosis of hepatic nodular lesions can be difficult. To differentiate HCC with MRI and CT scanning from the so-called regenerative nodules is especially difficult at a small size. C4.4A could be detected using a radiolabeled antibody by scintigraphy, thereby improving the sensitivity and specificity of non-invasive imaging and enabling the differentiation of C4.4A-positive HCC from C4.4A-negative regenerative nodules in cirrhotic livers. If nodular lesions are detected in radiologic imaging, C4.4A expression in scintigraphy would give rise to the probability for the presence of a HCC in contrast to regenerative nodules, that are negative for C4.4A and comprise of non-tumorous liver tissue. C4.4A expression also occurs in other tumor entities and wound healing, but the differentiation to HCC is easily possible due to the topographic localisation. As a differential diagnosis of C4.4A-positive hepatic lesions, liver metastases from colorectal cancer need to be considered. However, in these cases the primary tumor can be easily identified by endoscopy, plus liver metastases have a different behavior in MRI and CT imaging.

Alternatively, serum exosomes or exosomes in gallbladder secretion might be used for diagnosis in the future, as C4.4A is readily recovered in exosomes in pancreatic carcinoma (21). However, this diagnostic method is connected with considerable effort and is not yet a standard procedure.

Data provided here clearly show that C4.4A is a highly relevant marker for HCC that can be detected in tumor samples. This is of high clinical relevance because C4.4A as a diagnostic marker could be detected using a radiolabeled antibody by scintigraphy. Further studies and clinical trials are warranted for verification, especially to test the hypothesis that serum exosomes or exosomes in gallbladder secretion could be used to detect C4.4A.

Furthermore, rare expression of C4.4A in non-transformed cells suggests anti-C4.4A as an adjuvant therapeutics in cancer treatment, a first report with an anti-C4.4A antibody-drug conjugate revealing promising results (25).

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