Golgi phosphoprotein 2 (GOLPH2) expression in liver tumors and its value as a serum marker in hepatocellular carcinomas

Riener, M O; Stenner, F; Liewen, H; Soll, C; Breitenstein, S; Pestalozzi, B C; Samaras, P; Probst-Hensch, N M; Hellerbrand, C; Müllhaupt, B; Clavien, P A; Bahra, M; Neuhaus, P; Wild, P; Fritzsche, F; Moch, H; Jochum, W; Kristiansen, G

Riener, M O; Stenner, F; Liewen, H; Soll, C; Breitenstein, S; Pestalozzi, B C; Samaras, P; Probst-Hensch, N M; Hellerbrand, C; Müllhaupt, B; Clavien, P A; Bahra, M; Neuhaus, P; Wild, P; Fritzsche, F; Moch, H; Jochum, W; Kristiansen, G (2009). Golgi phosphoprotein 2 (GOLPH2) expression in liver tumors and its value as a serum marker in hepatocellular carcinomas. Hepatology: Epub ahead of print.

Postprint available at:
http://www.zora.uzh.ch

Posted at the Zurich Open Repository and Archive, University of Zurich.
http://www.zora.uzh.ch

Originally published at:
Golgi phosphoprotein 2 (GOLPH2) expression in liver tumors and its value as a serum marker in hepatocellular carcinomas

Abstract

Hepatocellular carcinomas (HCCs) and bile duct carcinomas (BDCs) have a poor prognosis. Therefore, surveillance strategies including sensitive and specific serum markers for early detection are needed. Recently, Golgi Phosphoprotein 2 (GOLPH2) has been proposed as a serum marker for HCC, but GOLPH2 expression data in liver tissues was not available. Using tissue microarrays and immunohistochemistry, we semiquantitatively analyzed GOLPH2 protein expression in patients with HCC (n = 170), benign liver tumors (n = 22), BDC (n = 114) and normal liver tissue (n = 105). A newly designed sandwich enzyme-linked immunoassay (ELISA) was used to analyze GOLPH2 levels in the sera of patients with HCC (n = 62), hepatitis C virus (HCV) (n = 29), BDC (n = 10), and healthy control persons (n = 12). By immunohistochemistry 121/170 (71%) of HCC showed strong GOLPH2 expression, which was significantly associated with a higher tumor grade (P = 0.01). A total of 97/114 (85%) BDCs showed a strong GOLPH2 expression which proved to be an independent prognostic factor for overall survival (P < 0.05). Serum levels of GOLPH2 measured by ELISA were significantly elevated in patients with HCC with underlying HCV infection (median 18 mg/L, P < 0.05) and patients with BDC (median = 14.5 mg/L, P < 0.01) in comparison to healthy controls (median 4 mg/L).

Conclusion: GOLPH2 protein is highly expressed in tissues of HCC and BDC. GOLPH2 protein levels are detectable and quantifiable in sera by ELISA. In patients with hepatitis C, serial ELISA measurements in the course of the disease appear to be a promising complementary serum marker in the surveillance of HCC. GOLPH2 should be further evaluated as a serum tumor marker in BDC on a larger scale.
GOLPH2 Expression in Liver Tumors and its value as a serum marker in Hepatocellular Carcinomas

Marc-Oliver Riener¹*, Frank Stenner²*, Heike Liewen², Christopher Soll³, Stefan Breitenstein³, Bernhard Cornelius Pestalozzi², Panagiotis Samaras², Nicole Probst-Hensch¹, Claus Hellerbrand⁵, Beat Müllhaupt⁶, Pierre-Alain Clavien³, Marcus Bahra⁷, Peter Neuhaus⁷, Peter Wild¹, Florian Fritzsche¹, Holger Moch¹, Wolfram Jochum⁸, Glen Kristiansen¹

¹Departments of Pathology, ²Oncology, ³Visceral & Transplantation Surgery, ⁴Institutes of Social and Preventive Medicine/Surgical Pathology, ⁵Department of Gastroenterology and Hepatology, University Hospital Zurich, Zurich, Switzerland. ⁶Department of Internal Medicine I, University of Regensburg, Regensburg, Germany. ⁷Visceral & Transplantation Surgery, University Hospital Charité, Berlin, Germany, ⁸Institute of Pathology, Kantonsspital St. Gallen, St. Gallen, Switzerland.  
* both authors contributed equally

marc-oliver.riener@usz.ch, frank.stenner@usz.ch, heike.liewen@usz.ch, christopher.soll@usz.ch, stefan.breitenstein@usz.ch, bernhard.pestalozzi@usz.ch, panagiotis.samaras@usz.ch, nicole.probst@ifspm.uzh.ch, claus.hellerbrand@klinik.uni-regensburg.de, beat.muellhaupt@usz.ch, pierre-alain.clavien@usz.ch, marcus.bahra@charite.de, peter.neuhaus@charite.de, peter.wild@cell.biol.ethz.ch, florian.fritzsche@usz.ch, holger.moch@usz.ch, wolfram.jochum@kssg.ch, glen.kristiansen@usz.ch

Keywords: Immunohistochemistry, Tissue-Microarray, serum, ELISA, Prognosis
Corresponding author:
Dr. Glen Kristiansen, MD
Institute for Surgical Pathology
Department of Pathology
University Hospital Zurich (USZ)
Schmelzbergstr. 12
8091 Zurich, Switzerland
Phone: +41 44 255 34 57
Fax: +41 44 255 44 16
Email: glen.kristiansen@usz.ch

Abbreviations: HCV, hepatitis C Virus; HCC, Hepatocellular Carcinoma; BDC, Bile Duct Carcinoma; ELISA, Enzyme-linked Immunosorbent Assay; AFP, alpha-fetoprotein; LCA, Liver-cell adenoma; FNH, Focal Nodular Hyperplasia; HBV, hepatitis B Virus; ICC, Intrahepatic Cholangiocarcinoma; ECC, Extrahepatic Cholangiocarcinoma; GBC, Gall Bladder Carcinoma; TMA, Tissue Microarray; PBS, phosphate buffered saline; RT, room temperature; TRACE, Time resolved Amplified Cryptate Emission; DMEM, Dulbecco’s Modified Eagle Medium; SDS, sodium dodecyl sulfate polyacrylamide; PVDF, Polyvinylidenfluorid; HEV, hepatitis E Virus; HDV, hepatitis D Virus; n.s., not significant.

Grant sponsor: Zurich Cancer League, Switzerland (H.M. & G.K.)
Abstract

Hepatocellular Carcinomas (HCC) and Bile Duct Carcinomas (BDC) have a poor prognosis. Therefore, surveillance strategies including sensitive and specific serum markers for early detection are needed. Recently, GOLPH2 has been proposed as a serum marker for HCC, but GOLPH2 expression data in liver tissues was not available. Using tissue microarrays and immunohistochemistry we semiquantitatively analysed GOLPH2 protein expression in patients with HCC (n=170), benign liver tumors (n=22) BDC (n=114) and normal liver tissue (n=105). A newly designed sandwich ELISA was used to analyse GOLPH2 levels in the sera of patients with HCC (n=62), HCV (n=29), BDC (n=10) and healthy control persons (n=12).

By immunohistochemistry 121/170 (71%) of HCC showed strong GOLPH2 expression, which was significantly associated with a higher tumor grade (p=0.01). 97/114 (85%) BDCs showed a strong GOLPH2 expression which proved to be an independent prognostic factor for overall-survival (p<0.05).

Serum levels of GOLPH2 measured by ELISA were significantly elevated in HCC patients with underlying HCV infection (median 18mg/L, p<0.05) and patients with BDC (median=14.5mg/L, p<0.01) in comparison to healthy controls (median 4mg/L).

Conclusions: GOLPH2 protein is highly expressed in tissues of HCC and BDC. GOLPH2 protein levels are detectable and quantifiable in sera by ELISA. In Hepatitis C patients serial ELISA measurements in the course of the disease appear to be a promising complementary serum marker in the surveillance of HCC. GOLPH2 should be further evaluated as a serum tumor marker in BDC on a larger scale.
1. Introduction

Hepatocellular carcinomas (HCC) and Bile-Duct Carcinomas (BDC) are the most common malignant tumors of the liver and they are frequently detected in advanced stages with a poor prognosis (1, 2). HCC often arises in the background of chronic liver disease and cirrhosis. In these patients surveillance strategies for the detection of early HCC are necessary. They include imaging techniques and serum markers such as AFP which has its limits (3). Therefore better markers are clearly needed (4). Recently GOLPH2/GP73, a Golgi apparatus associated protein, has been shown to have a higher sensitivity than AFP in the detection of HCC (5). Immunohistochemically GOLPH2 has been found in normal biliary epithelial cells whereas normal hepatocytes were negative (6). Furthermore GOLPH2 expression was significantly increased in liver disease due to viral causes (HBV, HCV) compared to nonviral causes (alcohol-induced liver disease, autoimmune hepatitis) (6). To our knowledge GOLPH2 protein expression has not yet been studied in tissues of liver tumors.

In this study, we performed a comprehensive GOLPH2 protein expression analysis in resection specimen of patients with HCC, liver-cell adenoma (LCA), focal nodular hyperplasia (FNH) and BDC using clinically well characterised tissue microarrays and correlated our findings with clinical-pathological parameters including patient survival. Further, a sandwich ELISA was created allowing serum level measurements and detection of anti-GOLPH2 antibody development in the serum. Applying this ELISA, sera from healthy individuals were compared to sera from patients with chronic hepatitis infections, HCC of various etiologies and BDC.
2. Material and Methods

Patients with hepatocellular tumors

One-hundred-seventy patients with HCC, 10 with LCA and 12 with FNH who underwent surgery between 1992 and 2007 in Zürich (n=110) and Regensburg (n=82) were enclosed in this study. In the HCC group the patients’ age ranged from 20 to 85 years (median 61 years). The underlying liver diseases of the HCC were: HBV-infection (27, 15.8%), HCV-infection (37, 21.7%), alcohol abuse (46, 27.1%), hemochromatosis (4, 2.4%), Alagille’s syndrome (1, 0.6%) and unknown etiologies (55, 32.4%). Of the 55 cases with unknown etiology, 30 (54.5%) suffered from concomitant liver cirrhosis. Corresponding non-neoplastic liver tissue of 100 patients with HCC, 8 patients with LCA and 9 patients with FNH was available. The median follow-up time of all patients was 17 months (range 1 to 120 months). Median follow up time of patients without disease progression was 17 months (range 1 to 120 months). Thirty-six percent of patients died during follow up after a median time of 14 months (range 1 to 79 months).

Patients with bile duct carcinoma

One-hundred-fourteen patients with bile duct carcinomas (BDC) who underwent surgery between 1995 and 2007 in Zürich were enclosed in this study. The BDC group consisted of 19 intrahepatic cholangiocarcinomas (ICC), 57 extrahepatic cholangiocarcinomas (ECC) and 38 gall bladder carcinomas (GBC). Patient age ranged from 36 to 90 years (median 64 years). Survival data was available for 109 patients. The median follow-up time of all cases was 9 months (range 1 to 86 months). Median follow up time of patients without disease progression was 11 months (range 1 to 58 months). Fifty-seven percent of patients died during follow up after a median time of 7.5 months (range 1 to 86 months).
Normal tissue

In order to evaluate GOLPH2 expression in normal liver and bile ducts five specimens of liver resections due to metastasis of colorectal carcinomas without specific liver disease were used. Five gall bladder resection specimens with mild chronic cholecystitis including normal epithelium were analysed for GOLPH2 expression in normal gall bladder epithelium.

Tissue Microarray

Formalin-fixed paraffin-embedded tissues were used to construct five tissue microarrays (TMA) with liver tumor tissues and two TMA with BDC tissues. The TMA was constructed as described previously (7). Two tissue cores per tumor with a diameter of 0.6 mm were punched out of the donor block and transferred to the recipient block.

Immunohistochemistry

The TMA blocks were freshly cut (3μm) and mounted on superfrost slides (Menzel Gläser, Braunschweig, Germany). Immunohistochemistry was conducted with the Ventana Benchmark automated staining system (Ventana Medical Systems, Tucson, AZ, USA) using Ventana reagents for the entire procedure. A monoclonal mouse antibody (clone 5B10, Abnova Corporation, Taipei, Taiwan, catalogue number H00051280-M06, dilution 1:1000) was diluted in a Ventana diluent, as recently described (8). The primary antibody was detected using the UltraVIEW DAB detection kit using the benchmarks CC1m- heat induced epitope retrieval. Slides were counterstained with hematoxylin, dehydrated and mounted. Prostatic tissue was used as a positive control.
Evaluation of the immunohistochemical stainings

The staining intensity of GOLPH2 was semiquantitatively evaluated with a four-tiered system: 0 (negative), 1+ (weak), 2++ (moderate), 3+++ (strong). Weak immunoreactivity was defined as minute granules projecting to the Golgi apparatus. Moderate immunoreactivity was diagnosed when a coarser and more intense staining was seen, whereas chunky, dark lumps were scored as strongly positive. A tumor was scored as positive when at least 10% of the tumor cells were stained. For statistical analysis, tumors were grouped into GOLPH2 weakly (0&1) and strongly (2&3) positive. The proliferation rate (MiB-1) was defined as percentage of positive nuclei per 100 tumor cells as recently described (9). The immunostainings were evaluated by two pathologists (MOR, GK) simultaneously on a multiheaded microscope.

Serological Tests

Serum samples (n =113, 62 HCC, 29 from patients with chronic hepatitis C infection (HCV), 10 BDC and 12 healthy volunteers) were collected from consented patients and healthy individuals.

Sera of patients were submitted to an ELISA for detection of serum GOLPH2 (sGOLPH2). The results were compared to serum values of healthy controls. The ELISA was performed as a sandwich ELISA as follows:

Maxisorb plates (NUNC, Langenselbold, Germany) were coated overnight at 4°C with 0.5 μg/ml polyclonal goat serum against GOLPH2 (Novus Biologicals; Littleton, CO, USA). The following day plates were washed with PBS and blocked using 10% Bovine Serum Albumin (BSA) (Sigma Aldrich; St. Louis, MO, USA) and 0.5% Tween 20 (Sigma) in PBS for 1 hour at room temperature. Serum samples were diluted 1:10 in PBS and applied in triplicate to the plate for 1.5 hours at room temperature. In some cases, due
to high concentrations of GOLPH2, further dilutions of sera were necessary (up to 1:500). Recombinant human GOLPH2 protein was plated in doubled increasing concentrations (0.025μg/ml, 0.05μg/ml, 0.1μg/ml, 0.2μg/ml, 0.4μg/ml, 0.8μg/ml, 1.6μg/ml, 3.2μg/ml, 6.4μg/ml) in triplicate. Plates were washed 5 times with PBS. The polyclonal rabbit serum against GOLPH2 (abcam; Cambridge UK) was employed for 1 hour at room temperature in a dilution of 1:1000 in PBS. After washing, a horseradish linked secondary anti-rabbit antibody (Sigma) diluted 1:10 000 in PBS was added for 45 minutes and plates were again washed extensively (7x PBS). Wells were replenished with 100 μl staining solution (TMB-solution) (Pierce, Thermo scientific, Rockford, IL, USA) per well and left at RT in the dark for 20 minutes. The colour reaction was stopped by adding 50μl 2 N H2SO4 to each well. The absorbance at 450nm was measured using an Emax microplate reader (Molecular device cooperation, CA, USA) and analysed with Softmax Pro V3.0 software. AFP serum levels were determined by TRACE (Time resolved Amplified Cryptate Emission).

Cloning and expression of recombinant human GOLPH2 protein

For the production of recombinant GOLPH2 protein to be used for reference measurements in the ELISA, the cDNA of GOLPH2 (ORF clone GOLM1 Transcript Variant1, Origene Technologies, Inc.; Rockville, USA) was amplified by polymerase chain reaction (PCR) using the following primer pair:

5´AAGAATTCGAGATGGGAAACG-3´ and 5´-AAAGGATCCGAGTGTATGATTCCGCTTTTCACG -3´. The PCR product was subcloned into the ECORI and BAMHI sites of pEAK8 (Edge BioSystems, Gaithersburg, MD, USA) that had been modified to contain the additional BAMHI and a 6 x histidine-tag in its multi-cloning site previously. The inserted cDNA was confirmed by complete sequencing.
The GOLPH2-HIS fusion product was transiently expressed in HEK-293 cells and detected by Western blotting in whole cell lysate and supernatant of these cells.

*Generation of stable cell lines for production of recombinant GOLPH2 protein*

HEK-293 cells were cultured in DMEM supplemented with 10% fetal calf serum, 50U/ml Penicillin 50ug/ml Streptomycin and 2mM L-Glutamin (Gibco Invitrogen; Carlsbad, USA). The cells were transfected with pEAK8-GOLPH2-HIS using Lipofectamin 2000 (Invitrogen; Carlsbad, CA, USA) according to the manufacturer’s recommendations. At 24 h postinfection, media of cells were supplied with puromycin. Remaining cells were subcloned and supernatants of clones expressing GOLPH2 were tested by immunoblots. A highly GOLPH2 expressing clone (H-G8 cells) was expanded and propagated for continuous production. Human recombinant GOLPH2-HIS protein was detectable in whole cell lysate and supernatant of H-G8 cells by ELISA and Western blotting (not shown). For purification supernatant of these stable cell line was dialysed against phosphate buffer (0.5M NaCl, 20mM Na Phosphate pH 7.3). His-tagged GOLPH2 was bound overnight on TALON metal affinity resin (Clontech; Mountain View, CA, USA). Washed resin (10 x resin bed volume with phosphate buffer) was eluted with increasing concentrations of imidazole (Sigma) from 15 mM to 250 mM and dialysed against PBS. Fractions were tested for GOLPH2 by Immunoblotting and for purity on Coomassie stained 10% SDS-Page gel. Concentration was measured using a Bradford assay (BioRad Laboratories AG; Reinach Switzerland).

*Statistical analysis*

Statistical analysis was performed using SPSS, version 15.0. Correlations were calculated according to Spearman. Fisher’s exact and chi-square tests were applied to assess the
statistical significance of the associations between the expression of GOLPH2 and various clinico-pathological parameters. Univariate survival analysis was carried out according to Kaplan-Meier, differences in survival curves were assessed with the Log rank test. Multivariate analyses were calculated according to the Cox regression model. P values < 0.05 were considered significant.

The study was approved by the local ethics committee (Kantonale Ethikkommission Zurich, StV 26-2005 and EK-1017).
3. Results

GOLPH2 protein expression in normal liver and bile duct tissue

Normal bile duct and gall bladder epithelium showed a weak GOLPH2 expression. The staining pattern was dot-like and localized perinuclear towards the cells apex projecting to the Golgi apparatus, while the cytoplasm was negative (Figure 1). A diffuse cytoplasmic GOLPH2 immunoreactivity that we have noted recently in a small subset of prostate cancer cases, was not seen. Interestingly single periportal hepatocytes were weakly positive while the rest of the hepatocytes were completely negative (Figure 1). In mesenchymal, stellate and endothelial cells we noted a weak to moderate dot-like GOLPH2 positivity similar to the description of Iftikhar et al. (10) and which we have also observed in other organs (Figure 1 and Figure 3) (8). In cirrhotic livers of different etiologies strong GOLPH2 expression in hepatocytes was found more frequently in patients with HCV infection (p<0.01, Table 1).

GOLPH2 protein expression in hepatocellular liver tumors and correlation with clinical-pathological parameters

121/170 (71.2%) of the HCC were strongly positive for GOLPH2 compared to 38/100 (38.0%) of the corresponding non-tumorous tissue (p<0.0001, Table 2). Strong GOLPH2 expression correlated significantly with a higher tumor grade (p<0.01) while no correlation with the other clinical-pathological parameters or survival was found (Supplemental Table 1, Supplemental Figure 1).

In contrast to normal liver tissue peritumoral tissue of LCA and FNH was strongly positive for GOLPH2 without a significant difference between tumor and non-tumorous tissue. 8/10 (80.0%) LCA were strongly GOLPH2 positive compared to 4/8 (50.0%) of the
corresponding peritumoral liver tissue while 4/12 (33.3%) of FNH compared to 1/9 (11.1%) of the peritumoral tissue (Supplemental Figure 2).

**GOLPH2 protein expression in BDC and correlation with clinical-pathological parameters**

17/19 (89.5%) of the ICC, 52/57 (91.2%) ECC and 28/38 (73.7%) GBC were strongly GOLPH2 positive (Figure 3). Due to the similarities in the GOLPH2 expression all these carcinomas were summarized as BDC. Taken together, 97/114 (85.1%) of these carcinomas showed a strong GOLPH2 expression. In this cohort GOLPH2 expression correlated with a younger patient age (p<0.01) while no correlation with other clinical-pathological parameters was found (Supplemental Table 2). Patients with BDC and GOLPH2 expression had a better overall survival as assessed by univariate analysis (p<0.001, Supplemental Figure 1). Further prognostic factors in our study group included patients age at diagnosis (<60 years vs. ≥60 years, p<0.05), higher pT-stage (pT1,2 vs. pT3,4, p<0.05) and R-status (p<0.001). In multivariate analysis including GOLPH2 expression, R-status, pT-stage and age, GOLPH2 expression was an independent prognostic factor for overall survival (p<0.05, Table 3).

**Analysis of GOLPH2 protein in Sera**

Levels of circulating GOLPH2 protein (sGOLPH2) were studied with a newly generated sandwich ELISA in sera of patients and control persons. When testing commercially available antibodies we found the combination of goat anti GOLPH2 for capturing the antigen and rabbit anti GOLPH2 for detection to be the most robust and reproducible design. Using this ELISA we measured sGOLPH2 levels in the range of 1.5 μg/ml to 60 μg/ml with 5% of sera exceeding the upper range.
The sGOLPH levels in this study are in the range of serum levels found for IL-2, IL-6, IL-8, TNF-α (11), CCSA-2 (colon cancer specific antigen 2) (12), Glypican-3 in HCC patients (13) or C-reactive protein (CRP).

Serum GOLPH2 levels of healthy controls were in the range of 1.5μg/ml to 20 μg/ml with a median of 4 μg/ml. Elevations were seen in patients affected by hepatitis C (median 9 μg/ml) and HCC of alcohol induced liver disease (AILD) etiology (median 7μg/ml). A more pronounced rise was found in patients with hepatitis C derived HCC (median 18μg/ml), especially if infected with HCV genotype 1b (median 19μg/ml) (Figure 4). In HCV positive HCC patients GOLPH2 serum levels were significantly higher than in sera of healthy controls (p<0.05). A non-significant trend towards higher values was seen in sera levels of HCC patients with a HCV-etiology compared to patients with a HCV-hepatitis (p=n.s.; Figure 4).

No correlation was noted between serum AFP and serum GOLPH2 levels (Figure 5). By setting the cut-off for sGOLPH2 at 10mg/L (above the 95% confidence interval of healthy references) and AFP at 10ng/ml approximately 30% of HCC cases exceeded this range for either GOLPH2 or AFP only. Both markers were elevated in 27% of cases and only 14% of patients were negative for both markers.

In the sera of patients with BDC (n=10) sGOLPH2 levels were significantly elevated (p<0.001)
4. Discussion

The data presented in this study provides further evidence that GOLPH2 protein is strongly expressed in HCC and BDC tissues and is secreted into the blood.

The 73kDa Golgi apparatus associated protein GOLPH2 is coded by the gene GOLM1 located on chromosome 9q21.33 and was originally cloned from a library derived from liver tissue of a patient with adult giant-cell hepatitis (14). GOLPH2 functions and the mechanisms of regulation in normal and neoplastic tissues are still unclear. Possibly it is either involved in posttranslational protein modification, transport of secretory proteins, cell signalling regulation or simply maintenance of Golgi apparatus function. GOLPH2 has several potential glycosylation sites and up to 75% of GOLPH2 secreted from hepatocytes is fucosylated (15). Endosomal trafficking of the normally membrane bound GOLPH2 leads to secretion into the blood making it a potential serum biomarker for HCC (16).

Surgical resection or liver transplantation are the only therapeutic options with curative intent for patients with HCC hence early detection is of utmost importance. Since most HCC develop in cirrhotic livers surveillance strategies for these patients have been employed including radiological and serological tests. One of the most commonly used approaches combines ultrasonography and serum AFP (sAFP) at an interval of 6-12 months (3). Unfortunately sAFP has a poor sensitivity, ranging from 39% to 65% and a specificity ranging from 76% to 97%. Therefore additional or better serum markers are needed (17). Recently, GOLPH2 was found to be up-regulated in sera of patients with HCC compared to healthy individuals and it was assumed that it might serve as a novel serum marker for HCC (5, 18). Unfortunately the authors found the applied immunoblot assay unsuitable for routine practice (5).
Previous studies have shown a better sensitivity of GOLPH2 than alpha-fetoprotein (5). In our study serum levels of sGOLPH2 and sAFP did not correlate, pointing to a diverse underlying cause of their elevation in HCC. Up-regulation of GOLPH2 was found in HepG2 cells after adenoviral infection suggesting that GOLPH2 is a marker for viral infection in liver tissue (6). This is in agreement with our findings of a significantly higher GOLPH2 expression in non-neoplastic liver tissue with HCV infection compared to other etiologies. In addition, most profound elevation of serum levels of GOLPH2 was detected in patients that had developed a HCC on the background of HCV genotype 1b infection. The control group with HCV genotype 1b infection without tumor had lower values in the ELISA test than the tumor bearing HCV infected patients but that difference did not reach significance. HCC of different etiologies like ethanol, other hepatitis subgroups including hepatitis C of other genotypes were not as clearly distinguishable by this method. However, due to the still limited number of serum samples analysed in this study, these results ought to be validated in a larger cohort.

The need for closer monitoring of chronically hepatitis infected individuals having a high risk of developing HCC during the course of the disease (19) has long been stated. In these patients sAFP has been a particularly unsatisfactory screening tool for early detection of HCC (20). From our preliminary experience we conclude that GOLPH2 is not a general HCC serum tumor marker but could rather be a valuable complementary tool in the surveillance of patients “at risk”. Further prospective studies are required to determine the predictive value of sequential measurement of serum AFP and sGOLPH2 as a new surrogate marker combination in the development of HCC.

Although GOLPH2 has been studied in normal liver and sera of patients with chronic liver disease and HCC, data about its protein expression in HCC tissue is lacking. In our study we found it highly expressed in the majority of the cases studied. Additionally it is
expressed frequently in the tissue of LCA and FNH and in the corresponding peritumoral liver tissue suggesting that it is not a specific marker for HCC.

Furthermore we found a high tissue expression of GOLPH2 in BDC. Interestingly, patients with high GOLPH2 expression in their tumors had a significantly better overall survival even in multivariate analysis, an unexpected finding that clearly warrants further confirmatory study. In the absence of established serum markers for BDC the raised sGOLPH2 levels in our ten BDC patients is an intriguing initial finding warranting further evaluation.

Increased GOLPH2 serum levels have been previously suggested as a specific finding for HCC. We have recently reported high tissue levels of GOLPH2 in adenocarcinomas of various organs including prostate, breast, colon (8) and some subtypes of renal cell carcinomas (21), which might confound the specificity of GOLPH2 as a serum marker for HCC. However, Block et al. did not find elevated sGOLPH2 levels in sera of nine patients with colorectal cancer (18) and Varambally et al. could not detect sGOLPH2 in serum of prostate cancer patients (22) using immunoblots. Still, more data is needed to clarify, if carcinomas other than HCC/BDC actively shed GOLPH2 into the serum.

While this paper was under revision a study showing similar results for serum GOLPH2 frequencies in liver diseases was published (23). Despite high concordance of our findings with regard to results and conclusions, Gu et al. measured approximately 100-fold lower absolute sGOLPH2 concentrations. This discrepancy is most likely due to methodological differences. The combination of the well characterised commercially available antibodies and a fully human recombinant standard protein presented herein may detect higher amounts of secondarily modified sGOLPH2 levels not recognized by a prokaryotic based detection system.
Taken together we found GOLPH2 protein highly expressed in HCC and BDC tumor tissues. Serum GOLPH2 measurements have promising potential for diagnostic and surveillance strategies of HCC patients.

5. Acknowledgments

We are grateful to Martina Storz, Silvia Behnke and Norbert Markuly for excellent technical assistance.
References


18. Block TM, Comunale MA, Lowman M, Steel LF, Romano PR, Fimmel C, Tennant BC, et al. Use of targeted glycoproteomics to identify serum glycoproteins that correlate


Figure legend

Figure 1. GOLPH2 Expression in normal liver, bile duct and gall bladder

A, B Perinuclear GOLPH2 positivity in bile duct (arrow) and in single periportal hepatocytes (arrowhead) while the majority of hepatocytes is negative. C Normal gall bladder weakly positive for GOLPH2. Mesenchymal cells weakly positive for GOLPH2 (arrow).

Figure 2. GOLPH2 expression in liver cirrhosis and hepatocellular carcinomas

A HCV induced liver cirrhosis negative for GOLPH2. B HCV induced liver cirrhosis strongly positive for GOLPH2. Hepatocellular carcinoma negative (C), weak (D), moderate (E) and strong (F) GOLPH2 positive.

Figure 3. GOLPH2 expression in bile duct carcinomas

Bile Duct Carcinoma negative (A) for GOLPH2 expression. Weak (B) and moderate (C) GOLPH2 granular positivity (arrow). Strong (D) GOLPH2 expression in clusters (arrow). Mesenchymal cells positive for GOLPH2 (arrowheads).

Figure 4A. Scheme of Sandwich ELISA

GOLPH2 depicted with the N-terminal proprotein convertase cleavage site and antibody binding sites (black aa 67-81, grey aa 302-402). Polyclonal goat anti GOLPH2 (black)) served as capture antibody. Polyclonal rabbit anti GOLPH2 (grey) secondary bound by a anti rabbit peroxidase labelled antibody (white asterisk) was used for detection.
Figure 4B. GOLPH2 sandwich ELISA.

GOLPH2 was detected by a sandwich ELISA in 113 serum samples, 12 healthy controls, 29 patients with HCV infection, 10 cases of bile duct carcinoma (BDC) and 62 patients with HCC. Out of 62 HCC patients 15 had alcohol-induced liver disease (AiLD), 18 were HCV positive. Displayed are boxplots of the different groups. An asterisk marks statistically significant difference revealed by the two tailed student’s t test between: Controls and HCC with HCV (p=0.038), controls and HCC with HCV 1b (p=0.014) and controls and BDC (p=0.0052)

Figure 5. Comparative analysis of GOLPH2 and AFP serum levels in HCC.

Dark green columns mark patients with elevated GOLPH2 but normal AFP. Light green columns mark cases with both GOLPH2 and AFP increased.

1-17: GOLPH2 elevated, AFP normal range (28%)
17-25: GOLPH2 and AFP normal range (14%)
26-43: GOLPH2 normal range, AFP elevated (31%)
44-59: GOLPH2 and AFP elevated (27%)

For individual patient HCC etiologies see supplemental Table 3 for case details of Fig.5
Supplemental Figure 1. GOLPH2 expression and survival in patients with hepatocellular carcinoma (HCC) and bile duct carcinoma (BDC)

Supplemental Figure 2. GOLPH2 expression in FNH and LCA

A Corresponding peritumoral liver tissue of FNH negative for GOLPH2. B FNH negative for GOLPH2. C Corresponding peritumoral liver tissue of LCA and LCA (D) strongly positive for GOLPH2.
Supplemental Figure 2