Metastasis-associated in colon cancer 1 is an independent prognostic biomarker for survival in klatskin tumor patients

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MACC1 is an independent prognostic biomarker for survival in Klatskin tumor patients

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List of abbreviations

CI, confidence interval; DFS, disease-free survival; G6PDH, glucose-6-phosphate-dehydrogenase; HGF, hepatocyte growth factor; HR, hazard ratio; ICC, intrahepatic cholangiocarcinoma; Klatskin tumors, hilar cholangiocarcinoma; MACC1, Metastasis-associated in colon cancer 1; OS, overall survival; qRT-PCR, Real time quantitative two-step RT-PCR; ROC, Receiver-Operating-Characteristics curve analysis.

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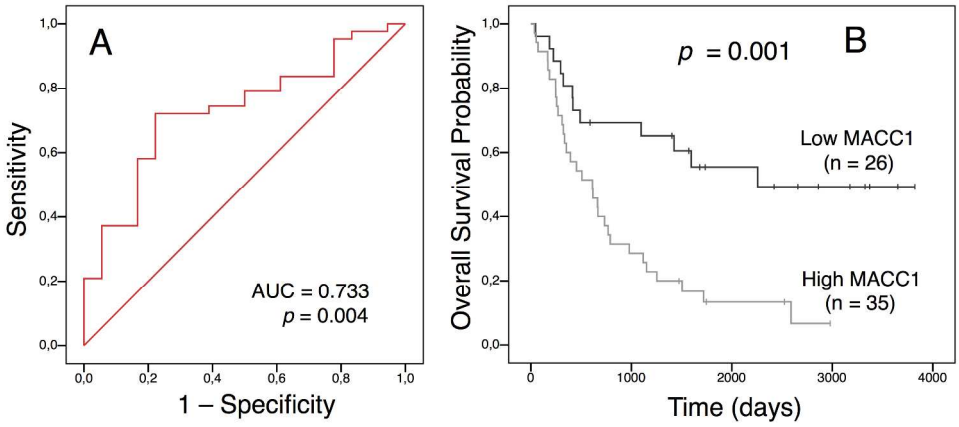
Abstract

Curative treatment of intrahepatic cholangiocarcinoma (ICC) and hilar cholangiocarcinoma (Klatskin tumors) is limited to surgical resection or orthotopic liver transplantation. However, not all patients benefit from a surgical approach and suffer from early tumor recurrence. Response to chemotherapy is generally poor and until today, no targeted therapy could be established. Metastasis-associated in colon cancer 1 (MACC1) is a recently discovered regulator of the HGF/Met/MAPK pathway, which induces proliferation, migration and invasion in cell culture, and metastasis in mice. MACC1 expression shows a significant correlation with Met expression in colon cancer tissue and is highly prognostic for the occurrence of distant metastasis and survival in colon cancer patients.

Thus, we aimed to measure the expression of MACC1, Met and HGF mRNA in microdissected tumor tissue and the corresponding normal liver tissue of 156 patients with Klatskin tumors (n=76) and ICC (n=80) using real time quantitative RT-PCR. We used immunohistochemical staining to validate the results. MACC1 expression in tumor tissue of both tumor entities was significantly higher than in corresponding normal liver tissue ($p<0.001$). Klatskin tumor patients with a history of tumor recurrence had significantly higher MACC1 expression than those without tumor recurrence ($p=0.005$). Univariate und multivariate survival analysis showed that Klatskin tumor patients with high MACC1 had a significantly shorter overall and disease-free survival ($p=0001$ and $p<0.001$, respectively). The multivariate analysis confirmed MACC1 to be an independent factor for overall survival in Klatskin tumor patients (HR 2.777; CI: 1.389-5.555; $p=0.004$).

Conclusion: Our study identified MACC1 as a highly prognostic biomarker for overall survival and disease-free survival in Klatskin tumor patients. MACC1 expression could become an important diagnostic tool and might be a candidate for targeted therapy.

Figure 3



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tumor recurrence and metastasis could be an essential tool to guide the treatment of patients with cholangiocarcinomas.

A multitude of growth factors and their respective signaling pathways have been described to be up-regulated in cholangiocarcinomas. These include the IGF/IGFR, EGF/TGF/EGFR, VEGF/PDGF/VEGFR, Wnt/β-catenin, and the hepatocyte growth factor (HGF)–Met pathway (8,9). The receptor tyrosine kinase Met is frequently overexpressed in cholangiocarcinomas, and Met expression has been linked to tumor differentiation and prognosis (10-13). However, the prognostic value of Met seems to differ between ICC and Klatskin tumors, and one study even showed a better patient outcome in case of Met overexpression in ICC (10,12).

Recently, our group discovered a new regulator of the HGF/Met/MAPK pathway, called Metastasis-associated in colon cancer 1 (MACC1). MACC1 was identified by a genome-wide search for differentially expressed genes in human colon cancer tissue, metastases, and normal tissue (14). The MACC1 gene is located on human chromosome 7 (7p21.1), the same chromosome that contains the genes of Met (7q31.2) and HGF (7q21.1). MACC1 turned out to be a powerful biomarker for the prediction of metachronous distant metastasis as well as survival in colon cancer patients. Based on the expression level of MACC1 mRNA, the negative and positive prediction of distant metastasis was 80% and 74%, respectively (14).

MACC1 mRNA expression in colon cancer tissue showed a significant correlation with Met mRNA expression. This was further investigated by in vitro studies, which revealed that MACC1 induces proliferation, migration and invasion in different colon carcinoma cell lines. Moreover, MACC1 promoted scattering in the presence of HGF. MEK inhibitors suppressed cell scattering, and vice versa, active MAPK promoted MACC1 expression. Furthermore, Met proofed to be a transcriptional target of

MACC1. Thus, MACC1 is a regulator of the HGF/Met/MAPK signaling pathway (14,15). This shows that MACC1 is not only a surrogate marker, but that it is causal for the development of distant metastasis, which makes it a highly interesting therapeutic target beyond its use as a prognostic biomarker (16).

Here we aimed at determination of the expression levels of MACC1, Met, and HGF in tumor and peritumoral tissue of ICC and Klatskin tumors. We investigate the relationship between MACC1, Met and HGF expression, and analyzed the prognostic significance of MACC1, Met and HGF expression for patient overall survival (OS) and disease-free survival (DFS). To the best of our knowledge, we present the first study to measure the expression level of MACC1, Met and HGF mRNA quantitatively in microdissected cholangiocarcinomas utilizing quantitative real-time RT-PCR technique (qRT-PCR) and immunohistochemistry.

Here we show for the first time, that MACC1 mRNA expression predicts OS and DFS in Klatskin tumor patients. This renders it highly interesting as a prognostic tool and makes it a potential therapeutic target for this type of tumor.

Experimental procedures

Human subjects

We obtained tumor specimens of 156 patients undergoing surgery between 1998 and 2003 with ICC (n=80) and Klatskin tumors (n=76) from the Department of General-, Visceral- and Transplantation Surgery, Charité Universitätsmedizin Berlin, Campus Virchow Klinikum, Berlin, Germany. Matching peritumoral liver tissue was available in all cases. In addition, we obtained normal bile duct tissue from 3 individuals undergoing surgery for benign liver tumors. All patients were treated by liver

resection. None of the patients received (neo-) adjuvant chemotherapy, and none of them had liver transplant. Patients with primary sclerosing cholangitis were not included in this study. The clinical, histopathological, and epidemiological data of the entire group of patients are reported in Supplementary Table 1. The follow-up period after surgery ranged from 0 days to 4007 days, with a median follow-up of 733 days. Collection of patient tissue and clinical data was approved by the local ethics committee (Charité Universitätsmedizin Berlin).

Tumor samples

Tissue specimens were collected during surgery, immediately snap frozen in liquid nitrogen and stored at -80°C. The investigators were blinded for patient metadata.

Histology and sample preparation

Serial sections were made of each tumor specimen for hematoxilin eosin (HE) staining, for microdissection with subsequent RNA isolation and for immunohistochemistry. Of each tumor sample, a section was HE stained following routine protocol. All HE stained sections were given to the Department of Pathology, Charité Universitätsmedizin Berlin, and were examined by a certified pathologist. Tumor diagnosis was confirmed and the respective tumor cell population on the slide was marked as a reference for microdissection. Samples with poor quality (e.g., necrosis) or samples lacking cancerous tissue were omitted from further analyses. With the exception of tumor diagnosis, the pathologist was blinded for all data, including tumor stage and patient outcome.

RNA extraction

Tumor samples were microdissected and the material was directly immersed into lysis buffer. Total RNA extraction was performed with the GeneMATRIX Universal RNA Purification Kit (Roboklon GmbH, Germany) according to the manufacturer protocol. Total RNA quality was assessed with a Bioanalyzer (2100 Bioanalyzer, RNA Pico Chips, Agilent, CA, USA) and concentration was measured with a NanoDrop spectrophotometer.

qRT-PCR

qRT-PCR was performed using the LightCycler 480 system (Roche Diagnostics). For each sample, a total of 50 ng RNA template was subjected to reverse transcription (RT) (MuLV Reverse Transcriptase, Applied Biosystems), and all subsequent qPCR reactions were done with the same RT reaction. Each qPCR reaction was carried out in duplicate with 5 ng cDNA template in a total reaction volume of 10 µl. For MACC1, Met, and for the house keeping gene glucose-6-phosphate-dehydrogenase (G6PDH), specific hybridization probes were used (synthesis TIB MOLBIOL, Berlin, Germany, and LightCycler hG6PDH Housekeeping Gene Set, Roche Diagnostics) with LightCycler FastStart DNA Master Hyprobe master mix (Roche Diagnostics). Detection of HGF mRNA was done with SYBR GoTaq qPCR Master Mix (Promega Corporation, USA). The respective primers (synthesis BioTeZ, Berlin, Germany) and hybridization probes used are shown in Supplementary Table 2.

Quantification was done with the standard curve method. Total RNA of cholangiocarcinoma cell line EGI-1 (for MACC1, Met, G6PDH) and of glioblastoma cell line HU 87 (for HGF) was diluted to generate a standard curve. For each point on the standard curve, a separate RT reaction with the respective amount of RNA

template was carried out. Additionally, a 50 ng RNA template of both cell lines was used as a calibrator, and the target gene expression was calculated as percentage of the respective calibrator sample. Standard curve and calibrator sample, as well as a no-template control were included in every qPCR run.

Immunohistochemistry

Immunohistologic staining for MACC1, Met and HGF was carried out for 20 specimens of each tumor entity. The samples were chosen based on normalized MACC1 qRT-PCR expression levels, whereas the specimens with the 10 highest and 10 lowest MACC1 expression levels were chosen. Fresh 5 µm cryosections were air dried for one hour at room temperature, fixed in 10 mM HEPES buffer containing 0,04% glutaraldehyde and 1% glucose, incubated with 0,9% hydrogen peroxide for 30 minutes, permeabilized with PBS containing 0,5% Triton X100 and 2,5% goat serum for 10 minutes, and treated with biotin blocking reagents (DAKO). After blocking with 5% goat serum for 45 minutes, the sections were incubated with the respective primary antibody at 4°C over night (please refer to Supplementary Table 3 for antibodies and dilutions). The sections were incubated with a biotinylated secondary antibody for 30 minutes and treated with a streptavidine peroxidase conjugate for 30 minutes (Strept ABC Complex, DAKO). The slides were developed with DAB for 1 minute (Liquid DAB+ Substrate, DAKO) and counterstained with hematoxilin. Sections without primary antibody served as controls.

The slides were examined with an Axioplan 2 microscope (Zeiss). Pictures of representative tumor areas were taken of each specimen at 200-fold magnification (Axiocam HRc camera, Zeiss). The same exposure and whitepoint setting was used for all slides. Pictures were evaluated using the Axovision 4.2 software (Zeiss) and a

semi-quantitative scoring system was applied. Tumor cells were classified to exhibit no staining (0), weak staining (1), intermediate staining (2), or strong staining (3). Analysis was carried out by two independent investigators who were blinded for the study protocol.

Statistical methods

Quantitative variables are expressed as medians. For comparison of groups, Kruskall-Wallis analysis on ranks and Mann-Whitney-U test were used. Inner group comparison was done using the Wilcoxon test.

Main study outcomes were DFS and OS of the patients. DFS was defined as the length of time after the primary operation the patient survived without sign of local recurrence or metachronous metastasis. OS was defined as length of time after the primary operation until patient death. Patients surviving less than 30 days after surgery were excluded from survival analysis to adjust for possible effects on survival related to surgical complications. For each tumor entity, median survival time and 95% confidence interval (CI) was calculated by Kaplan-Meier analysis. MACC1, Met, and HGF cut-off values were determined by Receiver-Operating-Characteristics curve analysis (ROC). ROC was carried out for every gene examined with the binary outcome variables for OS and DFS. The respective expression value yielding the highest *Youden* index was used as the cut-off value for univariate and multivariate survival analysis.

For the univariate survival analysis, categorical variables were tested using the log-rank test, and metric variables were tested with the Cox proportional hazard model. For the Cox proportional hazards model, the hazard ratio (HR) with 95% confidence interval was reported as an estimate of the risk of variable specific death. Variables

with p<0.1 in the univariate analysis were included in the multivariate analysis. The multivariate analysis was performed using the backward stepwise procedure for building a Cox proportional hazards model. Time-dependency was tested for every variable in the multivariate analysis and could be excluded. In all statistical tests, $P < 0.05$ was considered statistically significant. Statistical analysis was performed using IBM SPSS Statistics Version 20 (IBM Software Group, USA).

Results

MACC1 mRNA expression in Klatskin tumors and ICC is significantly higher than in corresponding normal liver tissue

MACC1, Met, and HGF mRNA expression could be detected in all tissues examined by qRT-PCR. MACC1 expression was significantly higher in Klatskin tumors compared to corresponding normal liver tissue, with 9.94 vs. 1.06 MACC1 mRNA expression/% calibrator ($p<0.001$; Figure 1A). MACC1 was also significantly higher expressed in ICC compared to corresponding normal liver tissue, with 8.98 vs. 0.75 MACC1 mRNA expression/% calibrator ($p<0.001$; Figure 1B). Met mRNA expression in Klatskin tumors was significantly lower compared to normal liver tissue (1.86 vs. 3.22 Met mRNA expression/% calibrator; $p=0.002$). Met mRNA expression in ICC did not differ significantly from the values measured in the corresponding normal liver tissue controls. HGF mRNA expression in all tumor tissues observed was significantly lower than in corresponding normal liver tissue: for Klatskin tumors 17.85 vs. 160.63, and for ICC: 20.82 vs. 187.60 HGF mRNA expression/% calibrator; all $p<0.001$.

The median expression level of MACC1 in biliary tumors was significantly higher than in the bile duct normal tissue controls, which showed MACC1, Met, and HGF mRNA expression levels comparable to normal liver tissue (Figure 1C).

We further investigated the expression levels of MACC1, Met, and HGF in selected histopathological subgroups (tumor size pT, lymph node involvement pN, metastasis pM, tumor grading G, and UICC stage) of each tumor entity. In the Klatskin tumor cohort, MACC1 mRNA expression was significantly higher in patients with a tumor

size of pT3 and pT4 than with pT1 and pT2, with 4.14 (pT1+2) vs. 8.03 (pT3+4) MACC1 mRNA expression/% calibrator (p=0.012; Figure 2A). MACC1 mRNA expression did neither differ significantly between the different UICC stages (Figure 2B), nor between the other major clinical features (pN, pM, and G). Met mRNA expression was also significantly higher in patients with a tumor size of pT3 and pT4 (1.27 vs. 2.19 Met mRNA expression/% calibrator; p=0.002) (Figure 2C).

No significant differences of Met mRNA expressions were found between the UICC stages and other histopathological categories in Klatskin tumor patients. For HGF mRNA expression, no significant differences could be detected for any of the analyzed parameters within the Klatskin tumor cohort.

In the ICC group, MACC1, Met, and HGF mRNA expression did not differ significantly between the histopathologic subgroups with respect to pT, pN, pM, and G category as well as UICC stage.

Survival analysis

MACC1 mRNA expression is a significant and independent prognostic marker for OS in Klatskin tumor patients

Next, we aimed to analyze the prognostic value of MACC1 expression in these tumor entities. ROC analysis was used to determine a cutoff value for OS and DFS. In total 61 out of 76 patients in the Klatskin tumor cohort survived more than 30 days after surgery and had complete clinical follow up. They were considered for survival analysis. In this group, ROC analysis for OS and DFS both yielded a significant area under the curve (AUC) for MACC1 mRNA expression (Figure 3A). Thus, the Klatskin tumor cohort was divided in group low and high MACC1 mRNA expression according to the cutoff value calculated by *Youden* index, which is 8.96 MACC1 mRNA

expression/% calibrator (sensitivity 70.8%; specificity 72%) (Figure 3A). Epidemiological, clinical, and histopathological data of Klatskin tumor patients grouped according to MACC1 mRNA expression status are reported in Table 3.

Univariate survival analysis

The median OS time in the Klatskin tumor cohort was 768 days (CI: 255-1251 days). Patients with high MACC1 expression had a median OS time of 613 days (CI: 300-926), which was significantly shorter than patients with low MACC1 expression (median survival time: 2257 days; CI: n/a; p=0.001) (Figure 3B). Survival analysis was repeated using the COX regression model with MACC1 as a binary as well as continuous variable. Both regression models confirmed the results of the log rank test. With MACC1 as continuous variable, the HR was 1.047 (CI: 1.024-1.072; p<0.001).

For Met mRNA expression, ROC analysis did not show a significant AUC. Using the median as cutoff value, univariate analysis showed Met mRNA expression to have a significant influence on OS. In cases with high Met mRNA expression, median survival time was reduced to 199 days (CI: 25-803 days), while patients with low Met mRNA expression had a median survival time of 340 days (CI: 588-1920 days; p=0.041). However, the effect of Met mRNA expression on OS was markedly weaker than for MACC1 mRNA expression. HGF mRNA expression had no significant influence on survival when analyzed with the log rank test. Using the COX regression model, Met and HGF mRNA expression had no significant effect on OS and DFS.

Another variable with significant influence on OS in the univariate analysis was the lymph node status with 1505 days for pN0 (CI: 870-2140 days) and 418 days for pN1 (CI: 224-612 days; p=0.011). Tumor size pT (p=0.07), occurrence of metastasis M

(p=0.209), tumor differentiation G (p=0.368), and resection margin R (p=0.169) were not significant parameters in our study. Patient age at surgery (p=0.545) and gender (p=0.950) had no significant effect on OS in the Klatskin tumor cohort.

Multivariate analysis for survival

We conducted a multivariate analysis to evaluate whether MACC1 mRNA expression is an independent factor for OS in the Klatskin tumor cohort. We used the COX regression model with the stepwise backwards procedure with all variables that had a significance of $p<0.1$ in the univariate analysis. Independent prognostic significance was detected for MACC1 expression with the ROC derived cutoff (HR 2.777; CI: 1.389-5.555; p=0.004) and lymph node status pN (HR 2.114; CI: 1.114-4.015; p=0.022). Met expression (p=0.601) and tumor size pT (p=0.296) were not significant. Therefore, we found MACC1 mRNA expression to be a strong and independent predictor of OS in Klatskin tumor patients.

MACC1 mRNA expression is a significant prognostic marker for DFS in Klatskin tumor patients

Patients with a history of tumor recurrence had significantly higher MACC1 mRNA expression than patients without tumor recurrence (Figure 4A). Moreover, patients with high MACC1 expression had a significantly shorter median DFS (753 days; CI: 341-1165 days) than patients with low MACC1 expression (>3119 days; CI: n/a; $p<0.001$) (Figure 4B). Met expression and HGF expression did not significantly influence DFS.

MACC1 mRNA expression is not a prognostic marker for survival of ICC patients

In the ICC group, 72 out of 80 patients were eligible for survival analysis. We were not able to generate a significant AUC for MACC1 mRNA expression with ROC analysis for ICC. In ICC, the AUC was 0.263, hence we did not use a ROC-derived cutoff value for survival analysis. Median survival time in the entire ICC group was 566 days (CI: 420–711 days). Using the median expression value (8.98 MACC1 mRNA expression/% calibrator) as cutoff, log rank test showed a significantly better survival in ICC patients with high MACC1 expression (median survival time: 1021 days; CI: 447–1595 days) than in patients with low MACC1 expression (median survival time: 437 days; CI: 293–581 days; p=0.002). Met (p=0.864) and HGF (p=0.295) expression showed no significant effect on OS when employing the median expression value as cutoff.

The univariate analysis for ICC showed a significant effect on OS time for tumor size pT (pT1+2: 1172 days; CI: 516–1828 days; pT 3+4: 440 days; CI: 310–570 days; p=0.006), and lymph node status pN (pN0: 881 days; CI: 327-1435 days; pN1: 357 days; CI: 296–418 days; p=0.006). Patient age at surgery (p=0.068), gender (p=0.216), metastasis M (p=0.237), tumor grading G (p=0.164), and resection margin R (p=0.415) were not significant variables for the prediction of OS time in the ICC group.

Correlation of MACC1 mRNA expression and Met mRNA expression

To further investigate the interrelationship between MACC1 and Met, we performed linear regression analysis for mRNA expression values in normal liver tissue and in tumor tissue on log transformed expression values. In Klatskin tumor tissue, there

was a strong correlation between MACC1 mRNA expression and Met mRNA expression (R=0.663, R^2=0.440, p<0.001). In contrast, there was no significant correlation of MACC1 and Met mRNA expression in normal liver tissue (R=0.225, R^2=0.051, p=0.056). In ICC tumor tissue, no correlation of MACC1 and Met mRNA expression could be detected.

Correlation of HGF mRNA expression and Met mRNA expression

Because HGF is the ligand for the Met receptor, we speculated that HGF and Met mRNA expression values might correlate better in normal liver tissue than in tumor tissue. For all tumor entities observed, we found a markedly higher correlation of HGF and Met mRNA expression in normal liver tissue than in tumor tissue using the linear regression model on log transformed expression values: Klatskin tumor tissue and normal liver tissue: R=0.325 *vs.* R=0.558, respectively; ICC tumor tissue and normal liver tissue: R=0.209 *vs.* R=0.719, respectively.

Validation of results obtained by qRT-PCR with immunohistochemistry

To validate the results obtained by qRT-PCR on the protein level, tissue samples were stained for MACC1, Met, and HGF. The results of semi-quantitative scoring of the staining intensity were plotted against the quantitative expression values measured by qRT-PCR. Spearman's rank correlation analysis showed a significant correlation between the results of qRT-PCR and immunohistochemistry (Figure 5). In all tissue specimens, staining for MACC1 was visible in the cytoplasm with some cell membrane staining, which was also the case for staining of Met. HGF staining was only visible in the cytoplasm. Representative pictures of immunohistochemistry are shown in Figure 6.

Discussion

In summary, here we report a first study measuring the expression for MACC1 mRNA, Met mRNA, and HGF mRNA quantitatively in microdissected cholangiocarcinomas. In our study, qRT-PCR was a suitable method to determine MACC1 expression in frozen tumor tissue. Most importantly, we identified MACC1 as a significant and independent prognostic biomarker for OS and DFS in Klatskin tumor patients.

The main goal of our study was to investigate whether MACC1 is of prognostic value in patients with ICC and Klatskin tumors. Additionally, we wanted to investigate the interrelationship between MACC1, Met, and HGF mRNA expression in these tumors and compare the prognostic usefulness between these genes. The scientific basis for this project was a study performed by our group, which identified MACC1 as a key regulator of the HGF-Met signaling pathway and demonstrated its ability to predict colon cancer metastasis not only when measured in solid tumors, but also when detected in human plasma (14,17). Therefore, we determined MACC1, Met, and HGF mRNA expression levels quantitatively using real-time RT-PCR in micro-dissected tumor tissue.

We found highly elevated MACC1 mRNA expression values in intrahepatic and hilar cholangiocarcinoma. MACC1 mRNA expression was approximately 10 times higher in intrahepatic cholangiocarcinoma and Klatskin tumors than in peritumorous normal liver tissue. Gene expression profliling in ICCs also showed elevated MACC1 expression levels (18). While the MACC1 expression in the Klatskin tumor cohort did not differ between the UICC tumor stages, we found significantly higher MACC1 expression values in patients with a tumor size of pT3 and pT4 compared to pT1 and

pT2. In addition, patients with a history of tumor recurrence had a significantly higher MACC1 expression than those without tumor recurrence. Although all Klatskin tumor patients with a poorly differentiated tumor (G3) were found in the high MACC1 mRNA expression group (Table 3), we found no significant difference in MACC1 mRNA expression between the different tumor grading categories. This finding is somewhat similar to the results reported on MACC1 expression in colorectal carcinoma, where MACC1 expression did not differ significantly between the different UICC stages but was significantly higher expressed in patients with metachronous metastasis (14,19).

Performing survival analysis, we found patients with low MACC1 expression having a highly significantly longer DFS time and a significantly longer OS time. To the best of our knowledge, this is the first report of a prognostic biomarker for Klatskin tumor patients.

It might seem controversial that, given the fact that MACC1 expression was elevated in ICC and Klatskin tumors alike, high MACC1 expression correlated only in the Klatskin tumor group with a poorer patient outcome. While both intrahepatic ICC and Klatskin tumors are neoplasms of the biliary system, they are clinically treated as different entities and show a different clinical behavior (20). This could be explained embryologically: Intrahepatic small bile ducts, which give rise to ICC, derive from hepatic progenitor cells. Klatskin tumors originate from the extrahepatic bile duct, which derives from the posterior ventral foregut together with the ventral pancreas (21). Furthermore, the signaling pathways active in these tumors are not the same, as was demonstrated by Mijamoto *et al.*, who found e.g. different expression levels of Met in intrahepatic and extrahepatic cholangiocarcinoma (10).

Met mRNA expression in Klatskin tumors was also of prognostic significance, although it was a markedly weaker biomarker and had no statistical significance in

ROC analysis. In Klatskin tumor tissue, we found a high correlation between MACC1 and Met mRNA expression. A significant correlation of MACC1 and Met expression has been reported for colorectal carcinoma, gastric carcinoma, and hepatocellular carcinoma (14,22-25). As MACC1 is a transcriptional regulator of Met, this suggests that MACC1 plays a pivotal role in carcinogenesis in Klatskin tumors. In ICC, no significant correlation between these genes was found. Furthermore, we found a significant correlation of HGF and Met expression in normal liver tissue. However, this correlation was lost in Klatskin tumor tissue, which might indicate that HGF as the ligand of Met is not the activator of the HGF-Met signaling pathway in these tumors. This is in accordance with studies in other tumor entities, which show a decline in HGF expression in case of Met being overexpressed (26).

Currently, the only curative therapeutic option available for Klatskin tumors is radical surgery with complete tumor removal (27-29). However, surgical resectability is often not given, and liver transplantation might seem a rational therapeutic approach to achieve complete tumor removal. Interestingly, in our patient cohort the only independent prognostic marker besides MACC1 expression was the lymph node status, which was also of prognostic significance in transplant trials for Klatskin tumors (7,30,31) The possibility of tumor recurrence is the major drawback for liver transplantation, and given the lack of biomarkers for the prediction of tumor recurrence, acceptable results for liver transplantation in Klatskin tumor patients are only feasible with a strict regime of preoperative staging and neoadjuvant chemoradiation (32,33). Consequently, liver transplantation for Klatskin tumors is only carried out in the setting of clinical studies. MACC1 could be a useful biomarker to identify a group of Klatskin tumor patients with a favourable clinical outcome after liver resection or liver transplantation.

Beyond its use as a biomarker, MACC1 is a promising candidate for targeted therapy. *In vitro* experiments targeting MACC1 with small hairpin RNA showed reduced tumor cell migration and invasion in colorectal, ovarian and hepatocellular carcinoma and *in vivo* experiments showed a reduction of tumor growth and metastasis formation in SW620 cell xenografts in mice by using MACC1 shRNA (14,34-36). The development of a selective inhibitor, for instance a small molecule targeting the MACC1 protein is a promising treatment modality for the prevention of tumor growth and tumor metastasis. This could be a potential adjuvant treatment for Klatskin tumor patients to reduce the risk of tumor recurrence.

Conclusion

In summary, our study identifies MACC1 as a highly prognostic biomarker for OS and DFS in Klatskin tumor patients. Prospective validation of MACC1 mRNA expression in Klatskin tumors may eventually make it a tool in clinical decision making to allocate patients suitable for curative surgery, which includes liver transplantation. Furthermore, down regulation of MACC1 might be an interesting therapeutic strategy to halt tumor progression.

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Figure legends

Figure 1

MACC1 mRNA expression in Klatskin tumors (A) and intrahepatic cholangiocarcinoma (B) with corresponding peritumorous normal liver tissue, and normal bile duct tissue (C), determined by qRT-PCR. Wilcoxon signed-rank test.

Figure 2

(A) MACC1 mRNA expression in Klatskin tumors with a size of pT1 or pT2 (median 4.14; n=16) and pT3 or pT4 (median 8.03; n=54), measured by qRT-PCR. (B) MACC1 mRNA expression in Klatskin tumor tissue of UICC stages I+II (n=63) and stages III+IV (n=5) as determined by qRT-PCR. (C) MET mRNA expression in Klatskin tumors with a size of pT1 or pT2 (median 1.27; n=16) and pT3 or pT4 (median 2.19; n=54), as determined by qRT-PCR. All Mann Whitney U Test.

Figure 3

(A) Receiver operator curve (ROC) for MACC1 mRNA expression with patient death as binary outcome variable in the Klatskin tumor group. AUC: Area under the curve. n=61. (B) Kaplan Meier analysis for OS of the entire Klatskin tumor cohort based on MACC1 mRNA expression level with ROC-derived cutoff value. Log rank test.

Figure 4

(A) MACC1 mRNA expression in Klatskin tumors from patients without tumor recurrence (median 5.25; n=50) and with tumor recurrence (median 14.77; n=24). Mann Whitney U Test. (B) Kaplan Meier analysis for DFS of the entire Klatskin tumor cohort based on MACC1 mRNA expression level with ROC-derived cutoff value. Log rank test.

Figure 5

Scatter graph: Semi-quantitative intensity score of immunhistochemical staining (no staining 0, weak staining 1, intermediate staining 2, or strong staining 3) for MACC1 and MACC1 mRNA expression measured by qRT-PCR in corresponding tissue samples of Klatskin tumor and ICC. Significant correlation was found with a Pearson and Spearman's rank correlation coefficient of R = 0,406 (p = 0,05) and R = 0,416 (p = 0,048), respectively.

Figure 6

Representative pictures of immunohistochemical staining for MACC1 in Klatskin tumor tissue. Staining intensity was classified as no staining (A), weak staining (B), intermediate staining (C), and strong staining (E). Immunohistochemical staining was done using the ABC method, counterstaining with hematoxilin. 200-fold magnification.

Table 1 Clinical features of the Klatskin Series (n=61) according to ROC based MACC1 grouping considered for survival analysis.

			MACC1		
Clinical features		**Total number of Cases**	**low (n=26)**	**high (n=35)**	***P*** †
Status					**<0,001**
	alive	18	14 (53.8)	4 (11.4)	
	dead	43	12 (46.2)	31 (88.6)	
Gender					0,395
	male	40	14 (53.8)	15 (42.9)	
	female	36	12 (46.2)	20 (57.1)	
Age		61	60±10	60±10	0,830
Size					0,744
	pT1	2	1 (3.85)	1 (3.03)	
	pT2	11	6 (23.07)	5 (15.15)	
	pT3	43	18 (69.23)	25 (75.75)	
	pT4	3	1 (3.85)	2 (6.07)	
Node Involvement					0,239
	pN0	33	17 (65.4)	16 (50)	
	pN1	25	9 (34.6)	16 (50)	
Metastasis					0,202
	pM0	57	26 (100)	31 (93.3)	
	pM1	2	0 (0)	2 (6.7)	
Histological Grade					**0,039**
	G1	2	1 (5.6)	1 (3.7)	
	G2	35	17 (94.4)	18 (66.7)	
	G3	8	0 (0)	8 (29.6)	
Resection Margin					0,806
	R0	38	17 (94.4)	21 (67.7)	
	R1	17	7 (5.6)	10 (32.3)	

Table values are given as mean ± SD for continuous variables and n (%) for categorical variables. † *P* value is for *t* test (continuous variable) or χ^2 test (categorical variables).

Figure 1

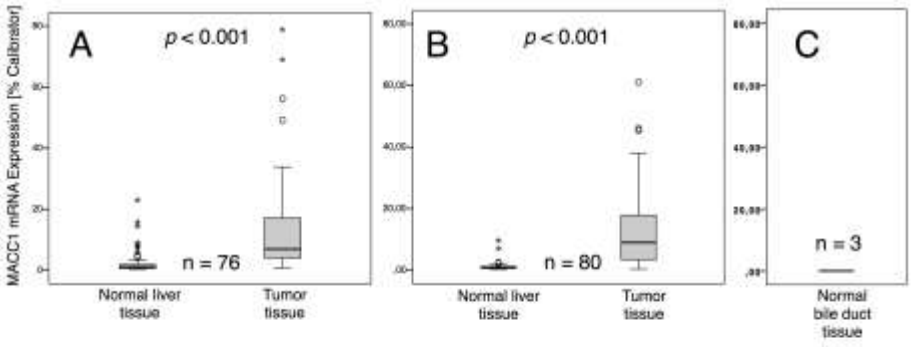

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Figure 2

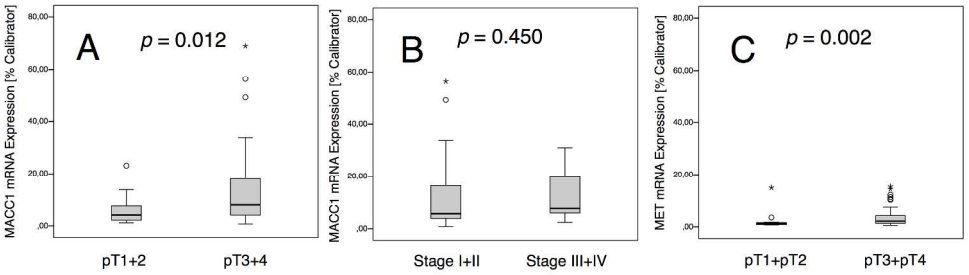

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Figure 3

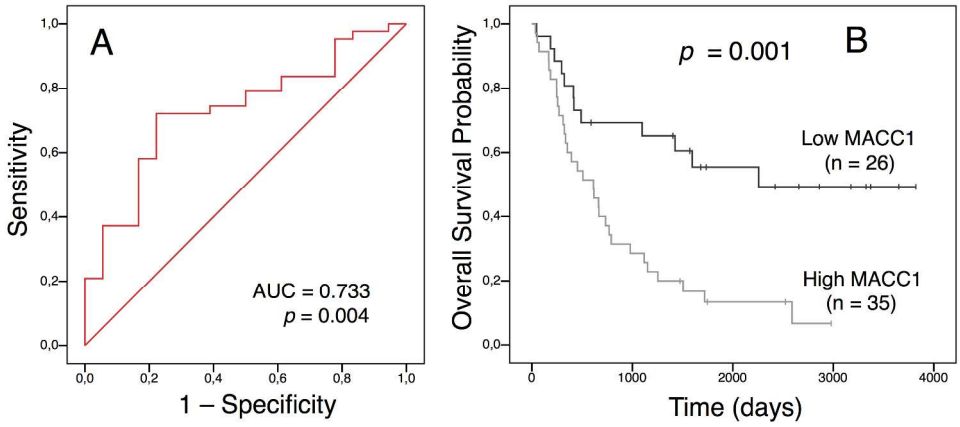

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Figure 4

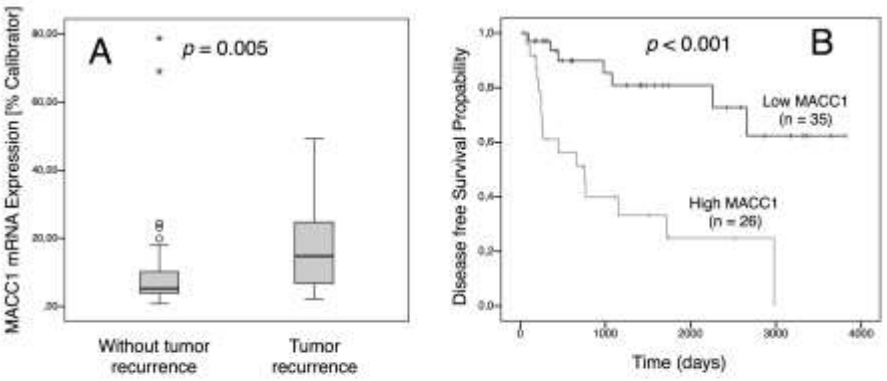

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Figure 5

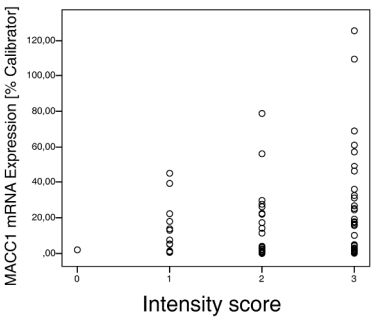

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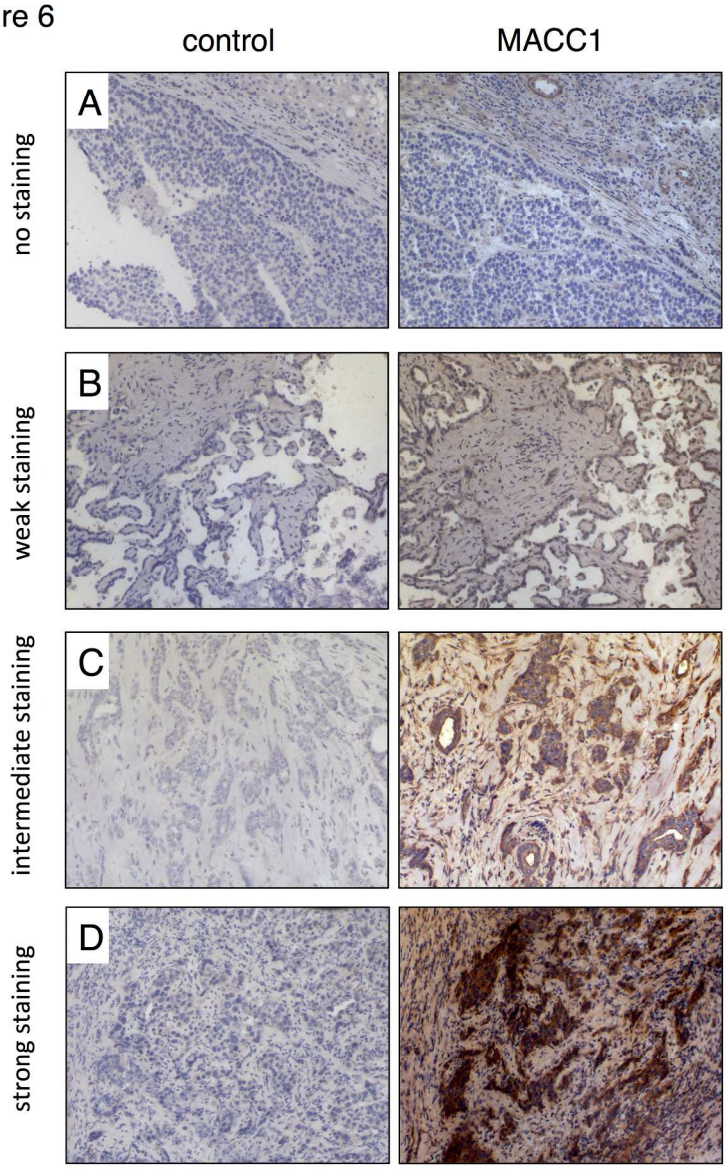
Figure 6



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Supplementary Table 1 Clinical features of the entire study Series (n=156) according to MACC1 grouping.

Clinical features		Total number of Cases	MACC1		*P* †
			low (n=73)	high (n=81)	
Status					0,501
	alive	31	16 (23,5)	15 (19,0)	
	dead	116	52 (76,5)	64 (81,0)	
Gender					0,132
	male	72	39 (53,4)	33 (41,3)	
	female	81	34 (46,6)	47 (58,7)	
Age		156	60±10	61±10	0,648
Size					0,454
	pT1	14	6 (8,6)	8 (10,8)	
	pT2	24	15 (21,4)	9 (12,2)	
	pT3	95	43 (61,4)	52 (70,3)	
	pT4	11	6 (8,6)	5 (6,8)	
Node Involvement					0,178
	pN0	72	36 (52,2)	36 (48,6)	
	pN1	71	33 (47,8)	38 (50,4)	
Metastasis					0,654
	pM0	136	65 (92,9)	71 (95,9)	
	pM1	8	5 (7,1)	3 (4,1)	
Histological Grade					0,770
	G1	2	1 (1,7)	1 (1,5)	
	G2	88	44 (74,6)	44 (67,7)	
	G3	34	14 (23,7)	20 (30,8)	
Resection Margin					0,899
	R0	72	34 (54,0)	38 (55,1)	
	R1	60	29 (46,0)	31 (44,9)	

Table values are given as mean ± SD for continuous variables and n (%) for categorical variables. † *P* value is for *t* test (continuous variable) or χ^2 test (categorical variables).

Supplementary Table 2 Primers and probes for quantitative real time PCR

Gene		Sequence
MACC1	Forward primer	TTC TTT TGA TTC CTC CGG TGA
	Reverse primer	ACT CTG ATG GGC ATG TGC TG
	FITC probe	GCA GAC TTC CTC AAG AAA TTC TGG AAG ATC TA
	Red640 probe	AGT GTT TCA GAA CTT CTG GAC ATT TTA GAC GA
Met	Forward primer	GAG AAG CCC AAG CCC ATC C
	Reverse primer	GCC CAG GGC TCA GAG CTT
	FITC probe	GCA GAC GAG CTG ATG AAG AGA GTG GGT TTC
	Red640 probe	AGT ATG AGG GCA CCT ACA AGT GGG TGA ACC
HGF	Forward primer	GGA CAA GAA CAT GGA AGA CT
	Reverse primer	ACA ACG AGA AAT AGG GCA AT
G6PDH	LightCycler hG6PDH Housekeeping Gene Set (Cat. No. 03261883001, Roche Diagnostics)	

Supplementary Table 3 Antibodies for immunohistochemistry

Antibody	Dilution
MACC1 (HPA020081, SIGMA)	1:250, 4°C over night
Met (sc-10, Santa Cruz Biotechnology)	1:400, 4°C over night
HGF (H-145) (sc-7949, Santa Cruz Biotechnology)	1:100, 4°C over night