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Title page

Differential expression of drug uptake and efflux transporters in Japanese patients with hepatocellular carcinoma

Tadashi Namisaki, Elke Schaeffeler, Hiroshi Fukui, Hitoshi Yoshiji, Yoshiyuki Nakajima, Peter Fritz, Matthias Schwab and Anne T. Nies

Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart, and University of Tübingen, Germany (T.N., E.S., P.F., M.S., A.T.N.)

Third Department of Internal Medicine, Nara Medical University, Japan (T.N., H.F., H.Y.)

Department of Surgery, Nara Medical University, Japan (Y.N.)

Department of Clinical Pharmacology, Institute of Experimental and Clinical Pharmacology and Toxicology, University Hospital, Tübingen, Germany (M.S.)

Running Title Page

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Address correspondence to:

Dr. Anne T. Nies

Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology

Auerbachstr. 112

D-70376 Stuttgart

Germany

E-Mail address: anne.nies@ikp-stuttgart.de

Telephone: +49-711-8101-3729

Fax: +49-711-85 92 95

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Nonstandard abbreviations:

ABC, ATP-binding cassette transporter; BCRP, breast cancer resistance protein; HCC, hepatocellular carcinoma; MDR, multidrug resistance; MRP, multidrug resistance protein; OCT, organic cation transporter; TACE, transarterial chemoembolization

Abstract

Targeted chemotherapy for hepatocellular carcinoma (HCC) is impaired by intrinsic and/or acquired drug resistance. Since drugs used in HCC therapy (e.g. anthracyclines or the tyrosine kinase inhibitor sorafenib) are substrates of uptake and/or efflux transporters, variable expression of these transporters at the plasma membrane of tumor cells may contribute to drug resistance and subsequent clinical response. In this study, the variability of expression of uptake (OCT1, OCT3) and efflux transporters (MDR1/P-glycoprotein, MRP1, MRP2, BCRP), selected for their implication in transporting drugs used in HCC therapy, was investigated. HCC and corresponding non-tumor tissue samples were collected from 24 Japanese patients at time of surgery. Protein expression was determined by immunohistochemistry. Expression data were correlated with clinicopathological characteristics and patients' outcome (median follow-up 53 months). Generally, expression was highly variable among individual tumor samples. Yet, median expression of OCT1, OCT3 and MDR1 in HCC was significantly lower (1.4-, 2.7- and 2-fold, respectively) than in non-tumor tissue, while expression of MRP2 persisted and BCRP showed a trend of increased levels in HCC. Patients with low BCRP expression had a significantly shorter overall and recurrence-free survival time. Results suggest different expression patterns of drug transporters in HCC, which are only in part associated with clinicopathological characteristics. Detailed information of expression of drug transporters in HCC may be promising for individualization and optimization of drug therapy of liver cancer.

Introduction

Hepatocellular carcinoma (HCC) is the 3rd leading cause of cancer mortality worldwide with highest incidence rates in Eastern Asia and the sub-Saharan region, regardless of recent improvement in early detection by imaging techniques as well as novel targeted therapy (El-Serag et al., 2008; Villanueva and Llovet, 2011; Forner et al., 2012; Llovet et al., 2012). Chronic hepatitis and cirrhosis, essentially associated with viral infection by hepatitis B or hepatitis C are the major underlying reasons for the development of HCC. Other risk factors are abnormal alcohol consumption, metabolic liver diseases (i.e. nonalcoholic steatohepatitis) or aflatoxin exposure. Irrespective of the etiology of HCC, only patients with early stage of HCC can benefit from radical therapies such as surgical resection, liver transplantation, or percutaneous local ablation. HCC patients diagnosed at intermediate to advanced stage are eligible for transarterial chemoembolization (TACE) or sorafenib therapy when residual liver function and physical status are preserved (Bruix and Sherman, 2011; Llovet and Bruix, 2008). Although targeted therapy for advanced HCC improves patient outcome, the management of HCC is limited by drug resistance as well as adverse drug reactions (Llovet and Bruix, 2008; Cheng et al., 2009).

Chemotherapy for HCC is impeded by the multidrug resistance (MDR) phenotype whereby tumor cells resistant to one anticancer drug are also resistant to drugs with completely different structures and mode of action (Baguley, 2010). Decreased drug uptake and increased drug efflux, mediated by integral membrane transporters, are considered as important mechanisms of drug resistance (Gillet and Gottesman, 2010). Many different transporters are physiologically expressed in human hepatocytes, which take up endogenous substances and drugs across the sinusoidal membrane and efflux them into bile (Degorter et al., 2012; Chu et al., 2013). In the context of HCC therapy the uptake transporters organic cation transporter 1 (OCT1,

encoded by *SLC22A1* gene) and 3 (OCT3, *SLC22A3*) as well as the ATP-binding cassette (ABC) efflux transporters MDR1 P-glycoprotein (*ABCB1*), multidrug resistance protein 2 (MRP2, *ABCC2*), and breast cancer resistance protein (BCRP, *ABCG2*) are of particular interest. They transport and confer resistance to anthracyclines, platinum drugs, and sorafenib (Cui et al., 1999; Burger et al., 2004; Yonezawa et al., 2006; Gillet and Gottesman, 2010; Herraez et al., 2013; Swift et al., 2013), which are commonly used in the treatment of HCC (El-Serag et al., 2008; Llovet et al., 2012). An increased expression of uptake transporters and a decreased expression of efflux transporters would favor the accumulation of cytostatic drugs within the tumor cells. Thus, patient-specific expression of uptake and efflux drug transporters may contribute to the optimization of the selection of HCC drugs and/or adjustment of dosing.

Several studies have already investigated drug transporter expression in HCC, however, either with focus on a single transporter, e.g. BCRP (Sukowati et al., 2012) or MDR1 (Ng et al., 2000; Kato et al., 2001; Akimoto et al., 2006), or several ABC drug efflux (Nies et al., 2001; Zollner et al., 2005; Sun et al., 2010) or OCT drug uptake transporters (Schaeffeler et al., 2011; Heise et al., 2012). An investigation comprising the simultaneous analysis of these drug transporters on protein level has not been performed so far.

Therefore, this study aimed (i) to systematically investigate protein expression of OCT1, OCT3, MDR1, MRP2, and BCRP in HCC and corresponding non-tumor samples from chemotherapy-naive patients with the same ethnic background and (ii) to evaluate drug transporter expression in association with clinicopathological characteristics and/or patient outcome.

Materials and Methods

Patients and liver tissues. Formalin-fixed paraffin-embedded samples of HCC and adjacent non-tumor liver tissue were obtained from 24 Japanese patients who underwent partial liver resection performed in the Department of Surgery, Nara Medical University Hospital, Japan, and Kokuho-central Hospital, Japan, between January 2000 to June 2009. The study was approved by the local ethics committee and all patients gave informed consent before surgery. HCC recurrence was assessed by ultrasound sonography, computed tomography, or magnetic resonance imaging every 2-3 months. One patient was excluded from analysis since he received pre-surgery chemotherapy (epirubicin). Seven patients received chemotherapy after surgery including epirubicin (n=4) and cisplatin as monotherapy (n=1), cisplatin in combination with 5-fluorouracil (n=1) or with 5-fluorouracil and epirubicin (n=1). None of the patients were treated with sorafenib. All HCC specimens were examined by an experienced pathologist and histological characterization was performed according to international criteria, i.e. grading (Hamilton and Aaltonen, 2000) and TNM stage (Singletary et al., 2003). Demographic and clinical data of the patients are summarized in Table 1.

Antibodies. Monoclonal antibodies were purchased, which specifically detected the following ABC efflux transporters: JSB-1 against MDR1 (Scheffer et al., 2000) was from Gene Tex Inc. (Irvine, CA); QCRL1 against MRP1 (Wright et al., 1998), M_2III-6 against MRP2 (Nies et al., 2001) and BXP-21 against BCRP (Zen et al., 2007) were from Alexis (San Diego, CA). Generation and use of the polyclonal antibodies KEN and CGR specifically detecting human OCT1 and OCT3, respectively, have been described previously (Nies et al., 2009; Schaeffeler et al., 2011).

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Immunohistochemical analysis. 3 µm paraffin sections mounted on glass slides (Dako Cytomation, Glostrup, Denmark) were deparaffinized with xylene (Merck, Darmstadt, Germany) and hydrated with 100%, 96% and 70% ethanol. Antigen retrieval was performed in citrate buffer 10 mmol/L, pH 6.0 (Dako) preheated to 99°C for 30 min. After blocking the endogenous peroxidase activity with peroxidase blocking solution (Dako) for 15 min, the sections were incubated for 30 min at room temperature with primary antibodies against MDR1, MRP2, OCT1, and OCT3 or overnight at 4°C with primary antibodies against BCRP and MRP1. Primary antibodies were diluted in a commercially available diluent (Dako) as follows: JSB-1 1:40, M_2III-6 1:20, BXP-21 1:50, QCRL1 1:20, KEN 1:4000 and CGR 1:200. Subsequently, the sections were incubated for 30 min at room temperature with goat secondary antibodies against mouse and rabbit IgG conjugated to peroxidase-labeled polymer (Envision+ system; Dako). The antigen-antibody immunoreaction was visualized with 3,3-diaminobenzidine tetrahydrochloride used as chromogen, followed by counterstaining with Mayer's hematoxylin. The slides were then dehydrated in alcohol and xylene and mounted with several drops of permanent aqueous mounting medium.

Evaluation of immunostaining. Immunohistochemical staining was evaluated by two independent observers with no knowledge of patient characteristics. Discrepancies were resolved by consensus. Intensity of membrane staining of tumor cells was assessed and scored as 0 (no staining), 1 (weak), 2 (medium) and 3 (strong) as described previously (Nies et al., 2001; Schaeffeler et al., 2011). Examples of different staining intensities are given in Supplemental Figure 1. By multiplying the intensity score and the proportion of immunopositive cells (0 to 100%), a semiquantitative

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staining score, ranging from 0 to 300, was calculated as previously described (Schaeffeler et al., 2011).

Statistical analysis. Data were analyzed using Prism software version 5.04 (GraphPad Software, Inc., La Jolla, CA). Data are expressed as medians and range. The Mann-Whitney test or the Kruskal-Wallis test were used as appropriate to calculate the effect of clinicopathological parameters (age, sex, tumor stage, tumor grade, tumor size) on drug transporter expression. Additionally, multivariate linear regression models were calculated with statistics software R-3.1.0 (R Core Team, 2014). To meet the Gaussian assumption expression data were log-transformed.

To analyze the impact of drug transporter expression on overall survival (OS) and recurrence-free survival (RFS), patients who showed expression levels <median were classified as "low expressors" vs "high expressors" (≥median expression). To analyze the effect of clinicopathological parameters on OS and RFS, patients were grouped according to the criteria given in Table 1. Kaplan-Meier curves were analyzed by log-rank Mantel-Cox tests. In order to account for a combination of expression of different transporters, Cox proportional hazard models were calculated with the survival plugin for R commander version 1.0-4 (Fox and Sá Carvalho, 2014). All tests were 2-sided and a P value <0.05 was considered to be significant.

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Results

Protein expression analysis of drug uptake transporters OCT1 and OCT3 and drug efflux transporters BCRP, MDR1, MRP2, and MRP1 in HCC. To systematically evaluate expression of drug transporters in HCC tissues, expression levels of OCT1, OCT3, BCRP, MDR1, MRP2, and MRP1 were analyzed by semi-quantitative immunohistochemistry as previously described (Schaeffeler et al., 2011). 91.3% (21/23 cases) of HCC samples showed lower OCT1 staining intensity (median: 140, range: 0-300) compared with the corresponding non-tumor tissues (median: 200, range: 100-300, Fig. 1A) resulting in 1.4 fold difference. As expected, OCT1 was localized at the basolateral membrane of hepatocytes in the non-tumor tissue (Fig. 1B) as well as of tumor cells, regardless of whether HCC had a trabecular (Fig. 1C) or pseudoglandular growth pattern (Fig. 1D). Similar to OCT1, OCT3 expression was significantly reduced by a factor of 2.7 in HCC (median: 60, range: 10-250) compared with non-tumor tissue (median: 160, range: 80-200; Fig. 1E). Distinct basolateral membrane staining was detectable in hepatocytes in the non-tumor tissue (Fig. 1F) and also in tumor cells of HCC growing in trabecular (Fig. 1G) or pseudoglandular pattern (Fig. 1H).

BCRP immunostaining score was higher in HCC than in matched non-tumor tissue in 73.9% of cases (17/23) leading to a 1.6-fold higher median BCRP staining score in HCC (median: 160, range: 20-270; Fig. 2A). BCRP was localized at the apical membrane of hepatocytes (Fig. 2B). In HCC, BCRP was also localized at the plasma membrane of tumor cells and staining appeared to be more intense on the apical site of the cells arranged in trabecular structures (Fig. 2C) and the luminal surfaces of those with pseudoglandular arrangements (Fig. 2D). The median staining score of MDR1 was significantly decreased by a factor of 2 in HCC (median: 50, range: 0-270) compared to

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non-tumor samples (median: 100, range: 50-200; Fig. 2E). Positive MDR1 immunostaining was found exclusively in the apical membrane of hepatocytes (Fig. 2F). In trabecular HCC (Fig. 2G) and in pseudoglandular HCC (Fig. 2H), MDR1 staining was found almost exclusively on the apical site of tumor cells. Median MRP2 staining intensity was not significantly different between non-tumor and HCC samples (P=0.513; Fig. 2I). Similar to localization of BCRP and MDR1, MRP2 was localized in the apical membrane of hepatocytes (Fig. 2J) as well as in the apical membrane of tumor cells that were arranged either in a trabecular pattern (Fig. 2K) or in a pseudoglandular pattern (Fig. 2L). In line with previous studies (Nies et al., 2001) immunohistochemical analysis revealed only occasional intracellular MRP1 staining in a few non-tumor and HCC cells (Supplemental Figure 2) and was therefore not quantified.

Correlation of drug transporter expression with clinicopathological characteristics. Correlations of transporter immunoreactivities and clinicopathological characteristics were assessed firstly by univariate analyses. Transporter immunoreactivities were not associated with age at surgery or sex (Table 2). However, significant associations were observed for OCT3 and BCRP, but not for OCT1, MDR1 and MRP2 (Table 2). In particular, a tumor size ≥ 20 mm was associated with decreased OCT3 and BCRP expression and, additionally, OCT3 expression was lower in higher tumor stage (Fig. 3). Moderate associations were observed between tumor grade and expression of BCRP, MDR1 (higher in G2) and OCT1 (lower in G2). By multivariate analyses, OCT3 and BCRP expression remained significantly associated with tumor size (Supplemental Table 1). Additionally, a significant relationship of OCT3 and BCRP expression with age and sex, respectively, was observed.

Relationship between drug transporter expression or clinicopathological characteristics and patient survival. Associations between drug transporter expression as well as clinicopathological characteristics with patient survival were assessed by calculating Kaplan-Meier survival estimates (Table 3A, Fig. 4). Median follow-up of the whole study population was 1604 days (range: 23-3331). BCRP expression was significantly correlated with OS and RFS, Table 3A, Fig. 4). Patients with low and high BCRP expression showed a median OS of 1517 days (range 23-1638 days) and 2847 days (range 1436-3331 days), respectively. RFS was 654 days (range 23-1515 days) in BCRP low expressors vs. 1684 days (range 561-2152) in high expressors. Since some of the patients received chemotherapy after surgery (n=7), we re-analyzed BCRP expression vs. clinical outcome only in patient without chemotherapy, again resulting in a significant correlation between high BCRP expression and better OS as well as RFS (Supplemental Table 2).

In contrast the expression of the uptake transporters OCT1 and OCT3 did not influence patient outcome, neither individually (Table 3A) nor in combination with each other (Table 3B) or with MDR1 (Table 3C).

Discussion

Resistance to drug therapy remains a major challenge in HCC treatment despite successful advances using targeted therapies (Llovet and Bruix, 2008; Cheng et al., 2009). Since the organic cation uptake transporters OCT1 and OCT3 (Yonezawa et al., 2006; Herraez et al., 2013; Swift et al., 2013) and the ABC efflux transporters MDR1, MRP2 and BCRP (Cui et al., 1999; Burger et al., 2004; Gillet and Gottesman, 2010) transport drugs being used in HCC therapy, protein expression of these transporters in HCC may be of interest with consequences for clinical outcome. For instance, doxorubicin and platinum compounds are used for TACE treatment of intermediate stage HCC and the tyrosine kinase inhibitor sorafenib for systemic treatment of advanced stage HCC (Llovet et al., 2012). While other studies have already investigated expression of either single transporters in HCC (e.g. BCRP [(Sukowati et al., 2012)]; MDR1 [(Ng et al., 2000; Kato et al., 2001; Akimoto et al., 2006)]) or only ABC or OCT drug transporters (Nies et al., 2001; Zollner et al., 2005; Sun et al., 2010; Schaeffeler et al., 2011; Heise et al., 2012), this study is the first to analyze simultaneously the expression of six efflux and uptake transporters with correlation to clinicopathological characteristics and clinical outcome. Although immuno-histochemistry used to determine protein expression is based on semi-quantitative scoring with some limitations, this technique is commonly used in clinical tumor diagnostics (e.g. estrogen receptor, Ki-67) due to a simple and cost-saving application.

Intrinsic resistance of various tumor entities to anti-cancer drugs already at onset of therapy has been linked to low expression of drug uptake transporters and/or high expression of drug efflux transporters in tumor tissue, thus indicating that the expression of those transporters are important contributors to drug response (Gillet and Gottesman, 2010). Therefore, we investigated the expression of the clinically relevant uptake transporters OCT1, OCT3 and the efflux transporters MDR1, BCRP and MRP2

in HCC in comparison to the corresponding non-tumor tissue. Of note, all our HCC samples were derived from chemotherapy-naive patients to exclude confounding of transporter protein expression by regulation (e.g. inhibition or induction) via chemotherapeutic agents such as anthracyclines and platinum compounds. Previous data have shown that indeed both drug classes may alter membrane transporter expression in cancer cell lines and in clinical samples (Sun et al., 2010; Herraez et al., 2012; Sukowati et al., 2012).

Our finding that OCT1 protein levels are significantly lower in HCC tissue than in non-tumor tissue from Japanese patients - mainly with hepatitis C etiology - is in line with previous reports on Caucasian HCC patients of various etiology (Schaeffeler et al., 2011; Heise et al., 2012). This indicates that OCT1 down-regulation appears to be a general feature of HCC irrespective of etiology or ethnicity. OCT3 protein expression was lower in HCC than in non-tumor tissue as well. To our knowledge, this is the first study systematically reporting on OCT3 protein levels in HCC tissue. Our previous observation that OCT3 protein expression is not decreased in a limited number of samples from Caucasian HCC patients (Schaeffeler et al., 2011) may be due to the fact that OCT3 expression – in contrast to OCT1 – is affected by etiology. Distinct gene expression patterns have been described for hepatitis B and hepatitis C positive HCC (Okabe et al., 2001). With regards to the drug efflux transporters BCRP, MDR1 and MRP2, their expression in HCC tissue in comparison to non-tumor tissue did not follow a uniform trend with up-regulation observed in some and down-regulation in other samples. Median expression in HCC compared to non-tumor tissue was lower (MDR1), higher (BCRP) or not changed (MRP2). This is in line with other reports (Sukowati et al., 2012; Zollner et al., 2005; Sun et al., 2010; Nies et al., 2001; Ng et al., 2000) and most likely reflects the molecular heterogeneity of HCCs (Thorgeirsson et al., 2006; Zollner et al., 2005).

Next, we attempted to identify whether clinicopathological characteristics may predict transporter expression in HCC tissue. Despite the small number of samples and the well-known molecular heterogeneity of HCC (Thorgeirsson et al., 2006; Zollner et al., 2005) our univariate and multivariate analyses showed that larger tumors are apparently associated with lower OCT3 and BCRP protein levels. Because sorafenib, which is used for systemic treatment of advanced stage HCC (Llovet et al., 2012), is effluxed by BCRP (Burger et al., 2004), we speculate that patients with larger tumors and hence decreased BCRP expression may benefit from treatment with sorafenib.

Finally, we observed that patients with high BCRP levels have had better outcome, taken into account that our study population was small and results were based on semi-quantitative immunohistochemical scoring. One function of BCRP is to protect cells against accumulation of harmful substances by pumping out environmental or endogenous toxicants (Huls et al., 2009). In case of high BCRP expression/function we suggest a protective role of BCRP for cells because BCRP actively effluxes dietary carcinogens such as the hepatocarcinogen aflatoxin B1 or heterocyclic amines (van Herwaarden and Schinkel, 2006). Moreover, BCRP has been shown to minimize cellular accumulation of porphyrins, including heme (Krishnamurthy et al., 2004). The regulation of intracellular porphyrin levels is important since excess levels may ultimately lead to generation of cell-damaging reactive oxygen species and to the collapse of mitochondrial function (Krishnamurthy et al., 2004). A protective role of BCRP for cells is supported by a study on intrahepatic cholangiocarcinoma - a subtype of liver cancer - in which better prognosis similar to our study was associated with high BCRP protein expression levels (Larbcharoensub et al., 2011). Mechanistically it is still unclear which underlying factors definitively contribute to better prognosis. However it has been recently shown that hepatic cancer stem cells are responsible for tumor relapse, metastasis, and chemoresistance (Yamashita and Wang, 2013). Indeed

immunohistochemical studies of stem cell markers suggest that HCC are histologically heterogeneous and contain a subset of cells expressing a variety of stem cell markers (e.g. Ma et al., 2007). Regarding intrahepatic cholangiocarcinoma it has been suggested that loss of BCRP expression renders progenitor cells prone to carcinogenesis and worsen the prognosis (Larbcharoensub et al., 2011). Thus, we suggest similar mechanisms to explain our association of better outcome and high BCRP expression.

Our study has some limitations. First, the study design was retrospective. Second, the number of HCC samples was small and a larger cohort is warranted to confirm our data. Finally, we did not investigate on whether genetic variation in the selected transporters may contribute to transporter expression in HCC since the number of samples was limited.

In summary, we investigated the expression of membrane transporters relevant for drugs used in HCC therapy. In our study including a limited number of HCC patients with Asian ethnic background we showed that the expression of selected uptake and efflux transporters (particularly BCRP) is highly variable. BCRP expression may be a predictor for patient outcome in HCC. To this end, our work highlights the need for comprehensive studies on transporter expression in cancer including interindividual variability and potential consequences for drug therapy.

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Authorship contributions

Participated in research design: Namisaki, Schaeffeler, Schwab, Nies

Conducted experiments: Namisaki, Schaeffeler, Nies

Performed data analysis: Namisaki, Schaeffeler, Fukui, Yoshiji, Nakajima, Fritz, Nies

Wrote or contributed to writing of the manuscript: Namisaki, Schaeffeler, Schwab, Nies

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Figure legends

Fig. 1. Expression of uptake transporter proteins in HCC and corresponding liver tissue. Immunohistochemical staining of drug uptake transporters OCT1 (A-D) and OCT3 (E-H) was performed on formalin-fixed and paraffin-embedded tissue samples from non-tumor tissue (B, F) and HCC tissue of trabecular (C, G) or pseudoglandular (D, H) growth pattern. Staining intensity was semi-quantified by considering the intensity of membrane staining of tumor cells and the proportion of immunopositive cells resulting in a staining score of 0-300. The median staining scores (blue horizontal lines) for OCT1 (A) and OCT3 (E) were significantly lower in HCC tissue than in non-tumor tissue (***, $P<0.001$). Bars, 20 µm.

Fig. 2. Expression of efflux transporter proteins in HCC and corresponding liver tissue. Immunohistochemical staining of drug efflux transporters BCRP (A-D), MDR1 (E-H) and MRP2 (I-L) was performed on formalin-fixed and paraffin-embedded tissue samples from non-tumor tissue (B, F, J) and HCC tissue of trabecular (C, G, K) or pseudoglandular (D, H, L) growth pattern. Staining intensity was semi-quantified by considering the intensity of membrane staining of tumor cells and the proportion of immunopositive cells resulting in a staining score of 0-300. The median staining score (blue horizontal line) for MDR1 (E) was significantly lower in HCC tissue than in non-tumor tissue (*, $P<0.05$) and that for BCRP (A) showed a trend for higher expression in HCC tissue ($P=0.056$). Bars, 20 µm.

Fig. 3. Association of drug transporter expression with tumor characteristics. Univariate analysis of drug transporter expression with tumor stage (A), tumor grade (B) and tumor size (C). OCT3 expression was significantly associated with tumor stage

and tumor size and BCRP expression with tumor size. Sample sizes were as follows: pT1: 9, pT2: 8, pT3: 6; G1: 15, G2: 8; size <20 mm: 6, size ≥20 mm: 17. *, $P<0.05$, ** $P<0.01$.

Fig. 4. Association of BCRP expression with survival (A) and recurrence-free survival time (B). Patients were stratified into two groups having BCRP expression levels <median (low BCRP expression, n=11) or ≥median (high BCRP expression, n=12). Kaplan-Meier curves were generated and analyzed using log-rank Mantel Cox tests. Patients with low BCRP protein expression had a worse survival (P=0.007) and a shorter recurrence-free time (P=0.002).

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Table 1

Clinical characteristics of HCC study population

Parameter	Category	n (% of total)
Age at surgery	< 60 years	3 (13.0)
	≥ 60 years	20 (87.0)
Sex	Female	5 (21.7)
	Male	18 (78.3)
Etiology	HCV	20 (87.0)
	HBV	1 (4.3)
	Alcohol	1 (4.3)
	Unknown	1 (4.3)
Tumor stage	pT1	9 (39.1)
	pT2	8 (34.8)
	pT3	6 (26.1)
	pT4	0
Histological grade	G1	15 (65.2)
	G2	8 (34.8)
	G3	0
Tumor size	< 20 mm	6 (26.1)
	≥ 20 mm	17 (73.9)
Chemotherapy after surgery	No	16 (69.6)
	Yes	7 (30.4)

HCV, hepatitis C virus; HBV, hepatitis B virus

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Table 2

Univariate analysis of drug transporter expression in relation to clinicopathological parameters

	***P* value**				
Parameter	**OCT1**	**OCT3**	**BCRP**	**MDR1**	**MRP2**
Age at surgery	0.169	0.216	0.581	0.747	0.314
Sex	0.368	0.911	0.084	0.652	0.601
Tumor stage	0.220	0.0095	0.119	0.711	0.057
Tumor grade	0.060	0.897	0.059	0.068	0.948
Tumor size	0.597	0.025	0.038	0.459	0.648

P values were determined using Mann-Whitney or Kruskal-Wallis tests as appropriate.

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Table 3

A Univariate correlation analysis of drug transporter expression and clinicopathological parameters with overall survival and recurrence-free survival

	Overall survival			**Recurrence-free survival**		
Parameter	*P* value	HR	95% CI	*P* value	HR	95% CI
BCRP expression	0.007	11.61	1.93-69.73	0.002	6.73	2.01-22.51
MDR1 expression	0.868	0.86	0.15-5.05	0.355	0.62	0.22-1.72
MRP2 expression	0.902	0.90	0.18-4.65	0.496	1.43	0.51-3.97
OCT1 expression	0.296	2.27	0.49-10.59	0.348	1.68	0.57-5.00
OCT3 expression	0.392	0.49	0.09-2.52	0.722	0.83	0.29-2.35
Age at surgery	0.362	0.31	0.03-3.82	0.752	0.80	0.20-3.20
Sex	0.573	1.72	0.26-11.35	0.236	0.50	0.16-1.58
Tumor stage	0.398			0.648		
Tumor grade	0.561	0.63	0.13-3.01	0.149	0.43	0.14-1.35
Tumor size	0.367	0.47	0.09-2.41	0.881	0.92	0.30-2.83
Chemotherapy after surgery	0.370	0.48	0.09-2.42	0.265	0.54	0.18-1.59

B Multivariate correlation analysis of OCT1 and OCT3 expression with overall survival and recurrence-free survival

	Overall survival			**Recurrence-free survival**		
Parameter	*P* value	HR	95% CI	*P* value	HR	95% CI
OCT1 and OCT3 expression	0.393	2.28 0.45	0.49-10.52 0.07-2.68	0.570	1.71 0.76	0.60-4.89 0.26-2.25

C Multivariate correlation analysis of OCT1, OCT3 and MDR1 expression with overall survival and recurrence-free survival

	Overall survival			**Recurrence-free survival**		
Parameter	*P* value	HR	95% CI	*P* value	HR	95% CI
OCT1 and OCT3 and MDR1 expression	0.600	2.28 0.45 0.93	0.49-10.53 0.07-2.69 0.15-5.62	0.620	1.64 0.80 0.64	0.57-4.75 0.27-2.41 0.21-1.90

P values were determined using log-rank tests (A) or Cox proportional hazard models (B, C). HR, hazard ratio; CI, confidence interval

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Figure 1

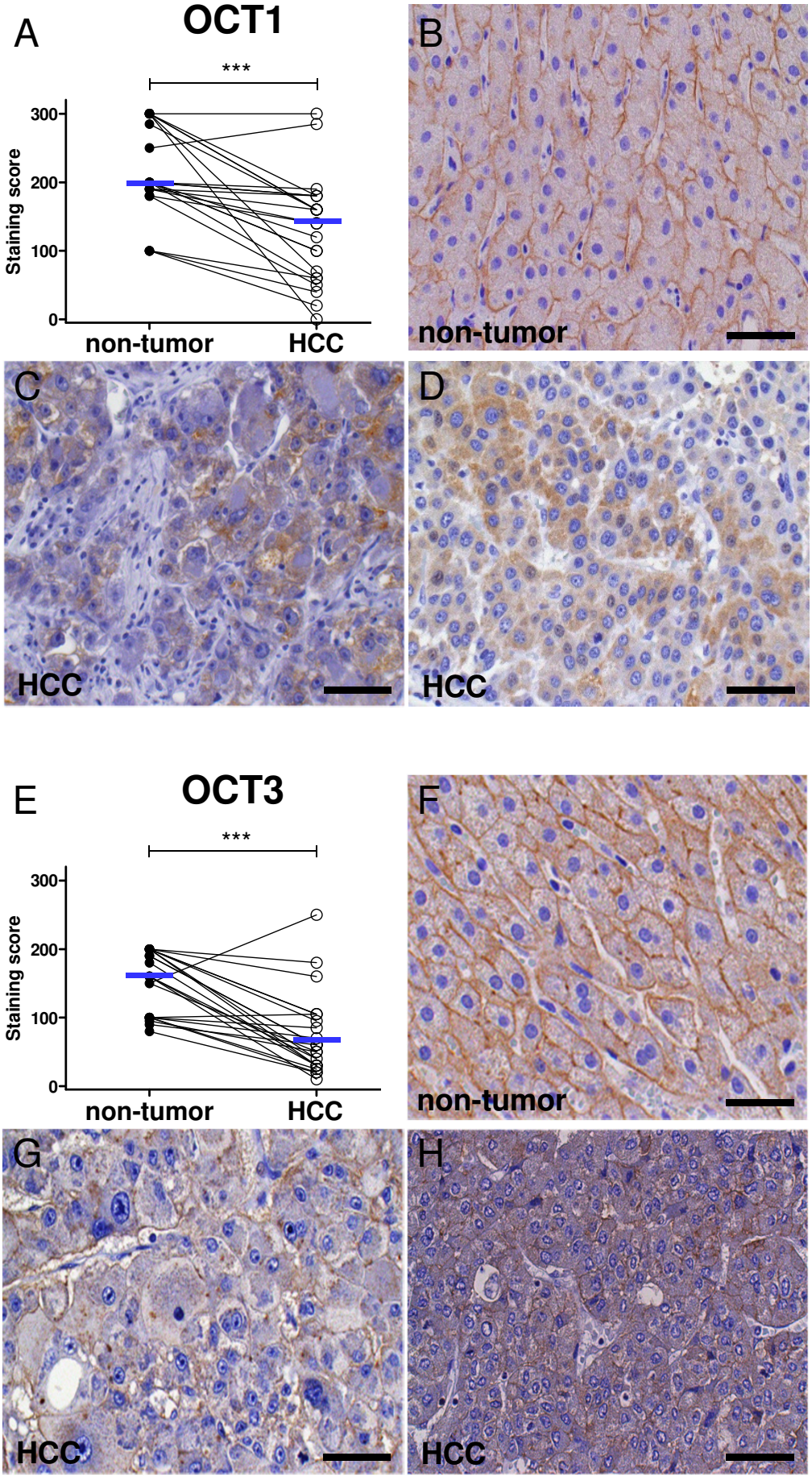

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Figure 2

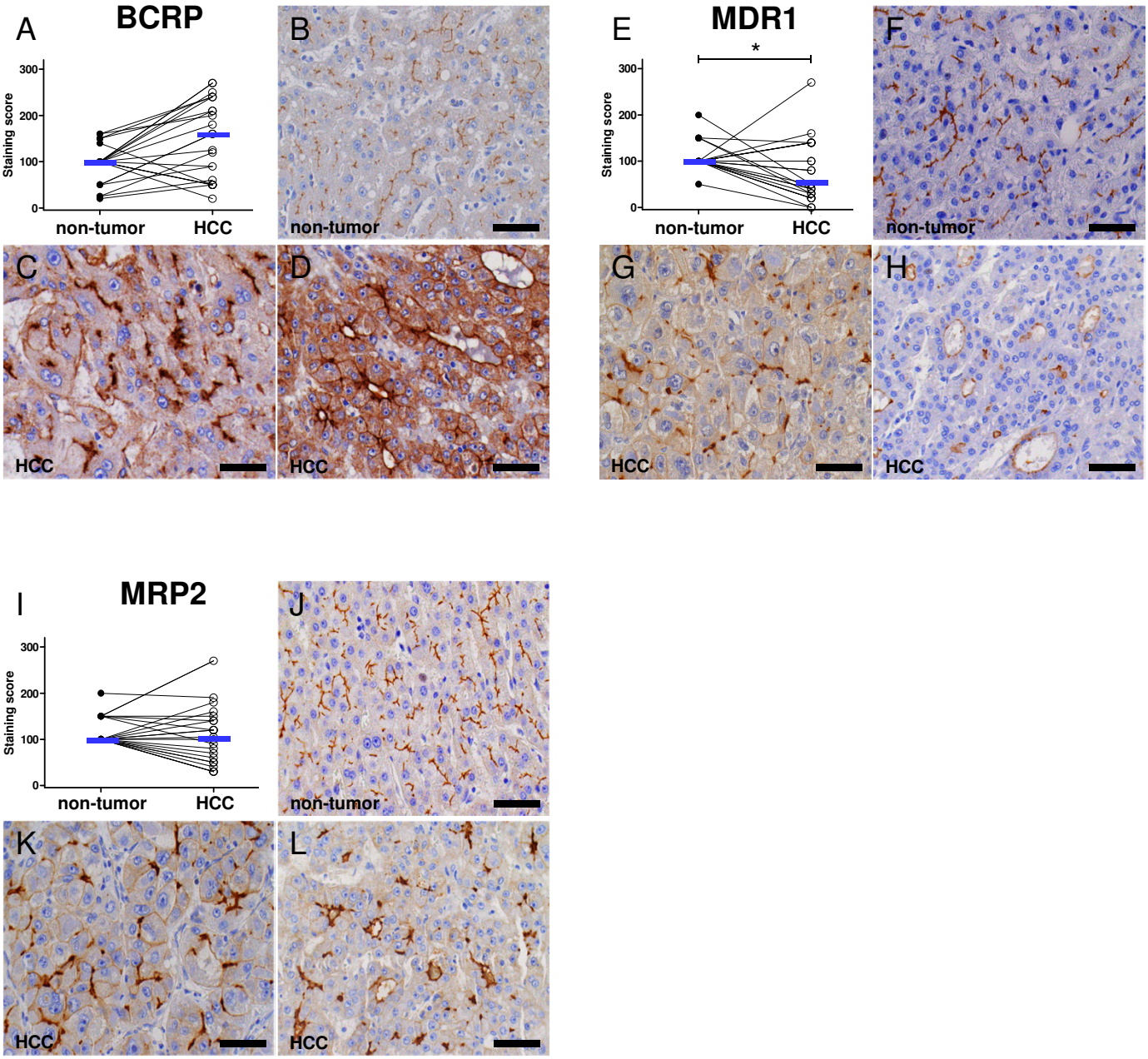

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Figure 3

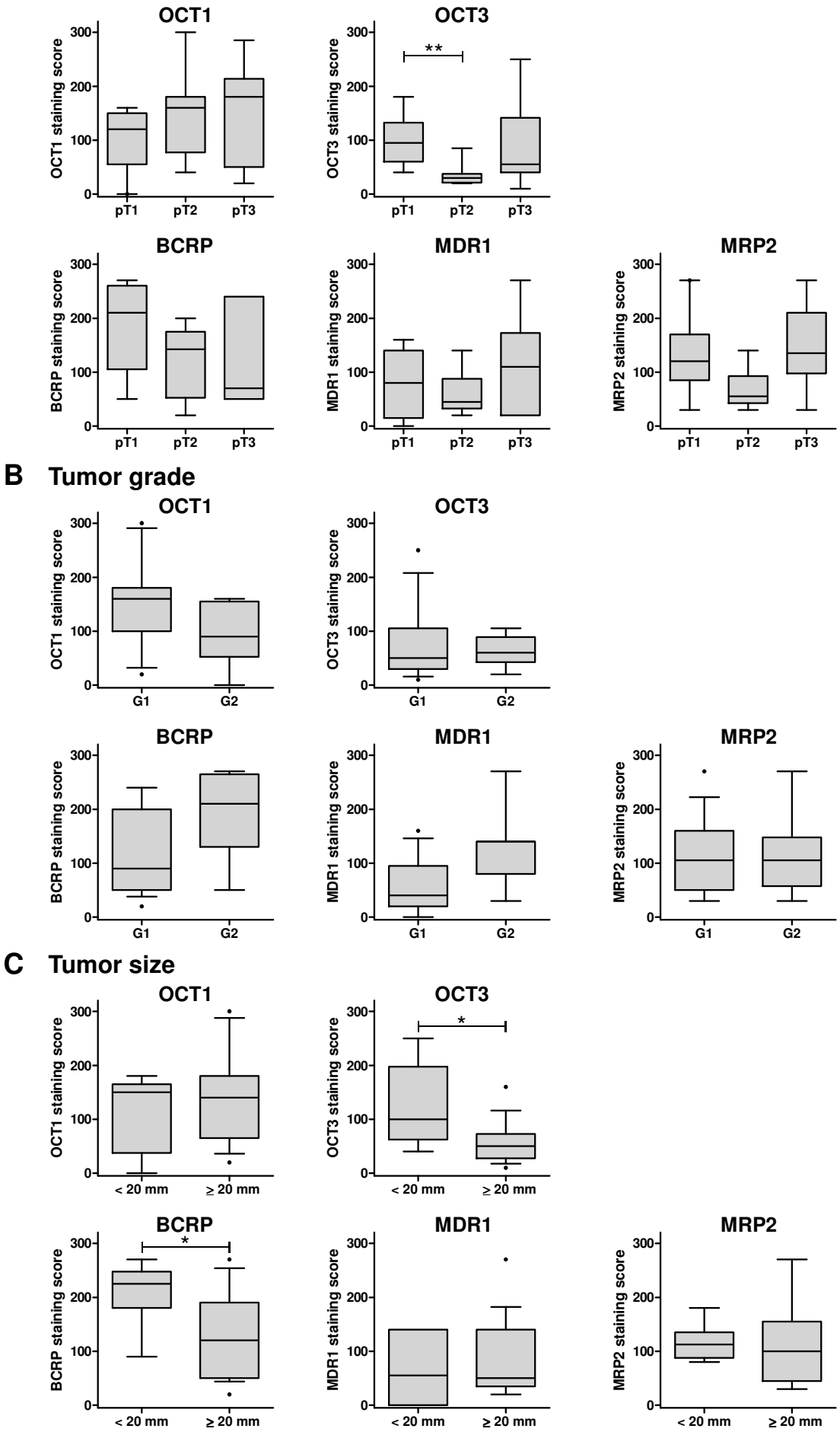

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Figure 4