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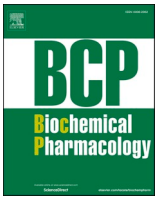

Role of organic cation transporter 3 (OCT3) in the response of hepatocellular carcinoma to tyrosine kinase inhibitors

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ABSTRACT

Impaired function of organic cation transporter 1 (OCT1) in hepatocellular carcinoma (HCC) has been associated with unsatisfactory response to sorafenib. However, some patients lacking OCT1 at the plasma membrane (PM) of HCC cells still respond to sorafenib, suggesting that another transporter may contribute to take up this drug. The aim of this study was to investigate whether OCT3 could contribute to the uptake of sorafenib and other tyrosine kinase inhibitors (TKIs) and whether OCT3 determination can predict HCC response to sorafenib. Cells overexpressing OCT3 were used to determine the ability of this carrier to transport sorafenib. Immunostaining of OCT3 was performed in HCC samples obtained in the TRANSFER study. Considering the intensity of staining and the number of OCT3-positive cells, tumors were classified as having absent, weak, moderate, or strong OCT3 expression and were also categorized according to the presence or absence of PM staining. Functional *in vitro* studies revealed that OCT3 is also able to mediate sorafenib uptake. Other TKIs, such as regorafenib, lenvatinib, and cabozantinib can also interact with this transporter. *In silico* studies suggested that the expression of OCT3 is better preserved in HCC than that of OCT1. In HCC samples, OCT3 was expressed at the PM of cancer cells, and its presence, detected in 26% of tumors, was associated with better outcomes in patients treated with sorafenib. In conclusion, analysis by immunohistochemistry of OCT3 in the PM of tumor cells may help to predict the response of HCC patients to sorafenib and potentially to other TKIs.

1. Introduction

After more than a decade in which sorafenib has been the only drug with effectivity, although moderate, in the treatment of advanced hepatocellular carcinoma (HCC), several novel drugs have been added to the pharmacological armamentarium in recent years, determining critical changes regarding recommendations for first, second and even third line of systemic treatment of these patients [1]. Nevertheless, sorafenib continues to be the first-line option for patients who are not recommended for combinations with immunotherapy (atezolizumab-bevacizumab or durvalumab-tremelimumab) and other tyrosine kinase inhibitors (TKIs), such as lenvatinib, regorafenib, and cabozantinib, are considered as options for first-, second- or third-line treatment [2–4].

The activity of these TKIs depends on their binding to the

Abbreviations: HCC, hepatocellular carcinoma; TKI, tyrosine kinase inhibitor; TKR, tyrosine kinase receptor; OCT, organic cation transporter; SLC, solute carrier; CCA, cholangiocarcinoma; OS, overall survival; FFPE, formalin-fixed, paraffin-embedded; CHO, Chinese hamster ovary cells; IHC, immunohistochemistry; FBS, fetal bovine serum; DHE, dihydroethidium; RT, reverse transcription; QPCR, quantitative PCR.

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intracellular domain of transmembrane tyrosine kinase receptors (TKRs). This implies that these drugs must reach the intracellular compartment of target cells to inhibit TKRs. The organic cation transporter 1 (OCT1, gene symbol *SLC22A1*) is essential for sorafenib uptake. Therefore, changes in the expression or activity of this transporter have been related to a reduced response to this TKI [5]. Besides, OCT1 downregulation has also been associated with tumor progression in HCC and cholangiocarcinoma (CCA) [6,7]. Decreased OCT1 expression in both types of liver tumors has been due partly to epigenetic modifications [8]. The level of *SLC22A1* mRNA in HCC samples was proposed as a potential biomarker for predicting response to sorafenib treatment [6]. Nevertheless, it was later demonstrated that the presence of OCT1 at the plasma membrane of tumor cells, rather than *SLC22A1* mRNA abundance, was related to a better outcome in HCC patients treated with sorafenib [9]. However, it was surprising that a small group of patients who did not express OCT1 in the plasma membrane of tumor cells still had an appropriate response to sorafenib as indicated by their prolonged overall survival (OS), which suggests that additional uptake mechanisms may be involved in plasma membrane crossing of sorafenib, and likely other TKIs. We know that OCT1 plays a key role in the uptake of sorafenib but not in regorafenib uptake [10], the drug that is administered second-line when resistance to sorafenib occurs.

Although in human hepatocytes the major organic cation transporter is OCT1, they also express OCT3 (gene symbol *SLC22A3*), and it has been described that both carriers have a considerable overlapping regarding substrate specificity, which under physiological circumstances ensures the performance of critical metabolic pathways in the event of dysfunction of one family member [11,12]. Owing to the impact of OCTs on the pharmacokinetic fate of therapeutically relevant drugs, both the FDA and the EMA recommend to screen compounds for possible interaction with OCT1, OCT2, and OCT3 [13]. These proteins belong to the *SLC22* family of the major facilitator superfamily of transporters. Whereas OCT1 and OCT2 are mainly expressed in liver and kidney, respectively, OCT3 is more widely located. Several genetic variants have been identified in the latter, which have been associated with dysfunctional changes in this transporter [14]. Thus, enhanced OCT3 expression has been described in HCC with very low *SLC22A1* mRNA levels [15]. While this reduced OCT1 expression in HCC has been associated with advanced stage, lost differentiation, and worse patient survival, these tumor characteristics were unrelated to *SLC22A3* mRNA levels [15]. Besides, the loss of OCT3 has been linked to enhanced proliferation and hepatocarcinogenesis in an experimental mouse model [16].

This study aimed to elucidate whether OCT3 can contribute to the uptake of sorafenib and potentially other TKIs used in the pharmacological treatment of patients with HCC, as well as to investigate if the analysis by immunohistochemistry of OCT3 alone or in combination with OCT1 in tumor samples can predict the outcomes of HCC patients treated with sorafenib.

2. Materials and methods

2.1. Human samples

Formalin-fixed, paraffin-embedded (FFPE) tissue specimens that had been collected between 2007 and 2011 from 39 patients treated with sorafenib in three German centers (University Hospital Würzburg, University Hospital Freiburg, and Hannover Medical School) as part of the TRANSFER [TRANsporter SoraFEnib Response] study [9] were used in the present study that was approved by the University of Salamanca IRB (PI2019-02-195) and conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent for the use of patient tissue samples was waived. Inclusion criteria were: i) Advanced HCC diagnosis based on pathology or imaging techniques obtained by dynamic contrast-enhanced multidetector CT scan or MRI according to European Association for the Study of the Liver (EASL) guidelines [17]; ii) Sorafenib therapy for at least four weeks with known outcome (radiological response and/or survival); iii) Compensated liver function prior to therapy (Child-Pugh Class A or B) and good Performance Status (PS 0–2); and iv) Time interval between tissue collection and start of sorafenib treatment no longer than 18 months (mean time interval was 13.6 ± 22.2 months; median = 7.1 months) [9]. Exclusion criteria were: i) No definite HCC diagnosis; ii) Other systemic chemotherapy between tissue collection and initiation of treatment with sorafenib; iii) Decompensated liver function (Child-Pugh Class C) or PS > 2 before initiation of sorafenib treatment.

2.2. Cells and chemicals

Chinese hamster ovary (CHO) K1 cells (CCL-61) were obtained from the American Type Culture Collection (ATCC) (LGC Standards, Barcelona, Spain). EGI-1 (ACC 385) cells, from human CCA, were obtained from the German Collection of Microorganisms and Cell Cultures collection (DSMZ, Braunschweig, Germany). CHO cells were cultured in DMEM medium with glutamax® solution and EGI-1 cells in DMEM/F-12 medium (Thermo Fisher, Madrid, Spain). Both media were supplemented with 10% heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin (Thermo Fisher), and in the case of CHO cells also with MEM Non-essential Amino Acid Solution (EGI-1) (Sigma-Aldrich, Merck, Madrid), amphotericin B (Thermo Fisher) and 0.43 mM L-proline (Sigma-Aldrich). CHO cells stably expressing OCT3 or OCT1 and EGI-1 cells stably expressing OCT3 were prepared by lentiviral transduction as previously described [10]. All the cells were routinely tested to ensure they were mycoplasma-free. Cabozantinib, lenvatinib, and regorafenib were from Selleckchem (Deltaclon, Madrid). Sorafenib tosylate was from Santa Cruz Biotechnology (Heidelberg, Germany). The purity of all these compounds was ≥98%. All other chemicals were of analytical grade.

2.3. Immunohistochemistry, immunofluorescence, and immunoblotting

Immunohistochemistry (IHC) was performed in the Pathology Service of the University Hospital of Salamanca using sections from FFPE material, with antigen retrieval at pH 6.0 and incubation for 40 min with OCT3 rabbit monoclonal antibody (Abcam, ab124826, Boston, USA) diluted 1:100 in a Leica Biosystems BOND-III Fully Automated IHC and ISH Stainer. Slides were counter-stained with hematoxylin (Palex, Madrid), mounted with aqueous mounting medium, and scanned at ×200 magnification using the dotSlide virtual image system (Olympus, Tokyo, Japan) at the Compared Molecular Pathology Service of the Salamanca Cancer Research Center (CIC). Images of the sections, made using Olympus software (DotSlide, OlyVIA, Olympus), were visualized by two independent investigators who were blinded to clinical data. A semi-quantitative histology score was calculated considering the intensity of the staining (absent, weak, moderate, and strong) and the percentage of stained cells. Tumors were also classified as positive or negative considering the localization of the staining at the plasma membrane. Immunofluorescence analysis was carried out on cryosections of air-dried tissue or cells grown on cover slides, which were fixed and permeabilized with ice-cold methanol. Primary antibodies were diluted in 2% FBS in PBS; OCT3 (Abcam, ab124826; 1:100), OCT1 (LifeSpan BioSciences, LS-C161155, Lynnwood, USA; 1:100), and MRP2 (Enzo Life Sciences, III2-5, NY, USA; 1:25). Appropriate secondary Alexa-488 or −594 antibodies (Life Technologies, Thermo Fisher) were diluted 1:1000. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Thermo Fisher). Confocal laser-scanning microscopy was performed using a Leica TCS SP2 confocal microscope.

Immunoblotting analyses of cell lysates prepared in radioimmunoprecipitation assay (RIPA) buffer were carried out in 7.5 % SDS-PAGE, loading 50 μg of protein per lane, which was previously mixed with loading buffer and β-mercaptoethanol and denatured by heating at 100 °C for 5 min, and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Blots were probed with primary monoclonal

antibodies against OCT3 (Abcam, ab124826; 1:1000), and GAPDH (clone 6C5, sc-32233, Santa Cruz Biotechnology, Dallas, USA; 1:1000) diluted in PBS-Tween with 3% (OCT1) or 5% (OCT3 and GAPDH) milk. Anti-rabbit (Abcam, ab6721) and anti-mouse (sc-516102, Santa Cruz Biotechnology) IgG-horseradish peroxidase-linked secondary antibodies were diluted 1:2000. Bands of immunoreactive proteins were visualized using enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Freiburg, Germany) in an image analysis equipment LAS-4000 (Fujifilm, TDI, Madrid).

2.4. Transport assays

Sorafenib uptake was determined as previously described [10]. In brief, cells were seeded in 6-well plates at a density of 75,000 cells/well, and experiments were performed one day later. The culture medium was removed and replaced with fresh medium containing 5 μM sorafenib. After 60 min, the uptake was stopped by rinsing the plates 4 times with 1 ml of ice-cold culture medium without FBS. Cells were then lysed using pure water containing 5 μM prednisolone (used as internal standard), and sorafenib concentration in the lysates was determined by HPLC-MS/MS in a 6420 Triple Quad LC/MS (Agilent Technologies, Santa Clara, CA, USA) following an adaptation of a previously described method [18]. The results were corrected by protein content. OCT1 and OCT3 transport activity was indirectly estimated as previously described [8] by competitive substrate uptake inhibition using the fluorescent compound dihydroethidium (DHE) as a typical substrate of both transporters. Briefly, cells were incubated with 2 μM DHE in the absence or the presence (10 μM) of a typical inhibitor (quinine) or one of the potential inhibitors to be tested. After 15 min of uptake period, the intracellular content of DHE was determined by flow cytometry.

2.5. Statistical analyses and in silico study

Student's *t*-test was used for comparisons between two groups. After analysis of variance, the Bonferroni method of multiple-range testing was used to calculate the statistical significance of differences among groups. The Log-rank test was used for the comparison of survival in Kaplan-Meier curves. When appropriate the Fisher's exact test was used.

An *in silico* study was carried out using information available in the Integrative Molecular Database of HCC (HCCDB), developed by Lian et al. [19], who have curated public HCC microarray and RNA-seq datasets, including The Cancer Genome Atlas Liver Hepatocellular Carcinoma Project (TCGA-LIHC) and the Genotype-Tissue Expression (GTEx), up to almost 4000 clinical samples. This resource has been compiled by Tsinghua University, National Center for Liver Cancer & Shanghai Eastern Hepatobiliary Surgery Hospital (China) and is available openly at http://lifeome.net/database/hccdb. The function *t*-test in R was used to detect whether there were significant differences in gene expression between tumor samples and adjacent samples in each dataset, followed by Benjamini-Hochberg correction [19].

3. Results

3.1. Ability of OCT3 to mediate the uptake of sorafenib and other TKIs

To investigate the ability of OCT3 to transport sorafenib, CHO cells previously transduced with empty lentiviral vectors (CHO-Mock) or with vectors containing the coding sequence of human OCT3 (CHO-OCT3) were used. The presence of OCT3 at the plasma membrane was assayed by immunofluorescence using a specific antibody, which showed no cross-reactivity with CHO-OCT1 cells, that had been previously obtained using a similar procedure (Fig. 1A-1F). Selective expression of OCT3 was also confirmed by immunoblotting in three separate transfection experiments (Fig. 1G).

The presence of OCT3 in CHO cells significantly increased sorafenib uptake compared with CHO-Mock cells (Fig. 1H). CHO-OCT3 cells were

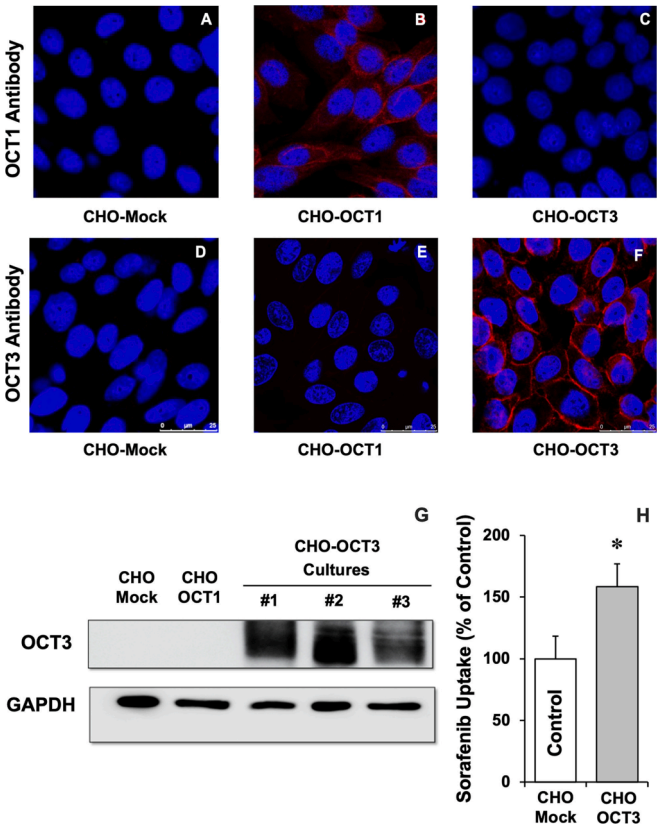

Fig. 1. Representative images of immunofluorescence analysis of OCT1 and OCT3 using specific antibodies (OCT1-Ab and OCT3-Ab, respectively) in Chinese hamster ovary (CHO) cells expressing OCT1 (CHO-OCT1) or OCT3 (CHO-OCT3) or transfected with empty lentiviral vectors (CHO-Mock) (A-F). Representative immunoblots of OCT3 and GAPDH in lysates obtained from these cells. Three different transfection experiments were used in the case of CHO-OCT3 cells (G). Sorafenib uptake by CHO-Mock or CHO-OCT3 cells was determined by HPLC-MS/MS after incubating the cells with 5 μM sorafenib for 1 h. Values are mean ± SD from data collected in 9 different wells from three separate experiments. *, $p < 0.05$ by the Student's *t*-test (H).

also used to determine the ability of other TKIs to inhibit the uptake of DHE, a known substrate of this transporter. As expected, quinine, a typical OCT3 substrate, competed with DHE reducing net uptake by CHO-OCT3 cells (Fig. 2C), obtained from subtracting the uptake of DHE by CHO-Mock cells (Fig. 2A) from the uptake by OCT3-overexpressing cells (CHO-OCT3) (Fig. 2B). Inhibition was also induced by several TKIs with the following order of magnitude in their effect: quinine > sorafenib ≈ cabozantinib > lenvatinib > regorafenib (Fig. 2C). The ability of these drugs to inhibit OCT3-mediated transport was confirmed in a different cell line (EGI-1). These CCA-derived cells were used because they also lack endogenous OCT3 expression (data not shown). The forced expression of OCT3 in these cells resulted in an enhanced ability to take up DHE (Fig. 2E) compared with DHE uptake in EGI-1-Mock cells (Fig. 2D). As shown in Fig. 2F, the increased DHE uptake mediated by OCT3 was inhibited by quinine > sorafenib > cabozantinib ≈ lenvatinib > regorafenib.

3.2. In silico study

To confirm previous reports [15] suggesting that the expression of OCT3 is relatively well preserved in HCC, especially when OCT1 expression decreases, which could be of interest to identify other patients who could benefit from sorafenib treatment, *in-silico* analysis of transcriptomic data from 11 databases compiled in the Integrative Molecular Database of HCC (HCCDB) was performed. Besides a marked

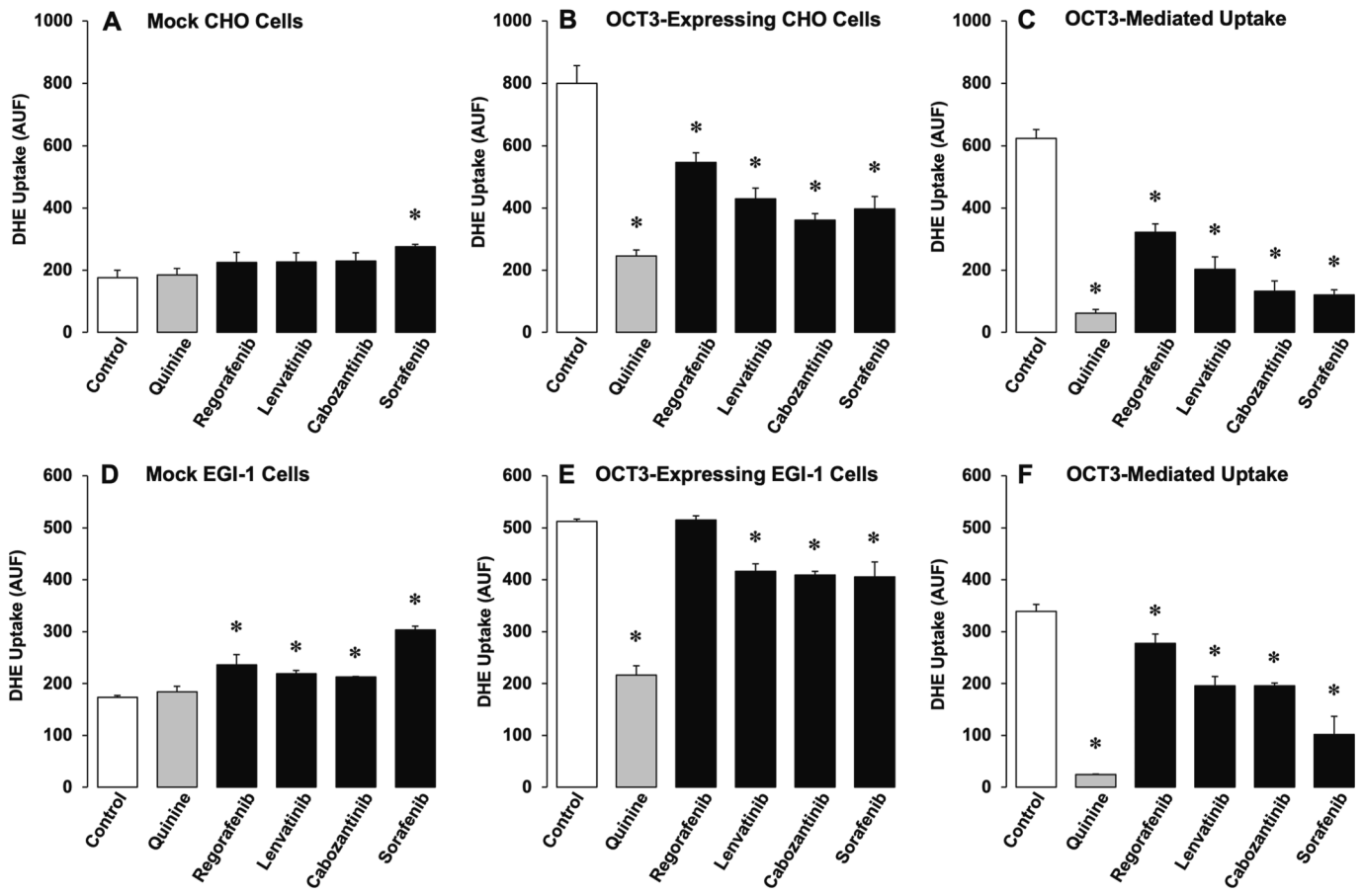

Fig. 2. Inhibition of dihydroethidium (DHE) transport by tyrosine kinase inhibitors used in the treatment of hepatocellular carcinoma. DHE uptake (2 μM, 15 min) by CHO cells transfected with empty lentiviral vectors (CHO-Mock) (A), CHO expressing OCT3 (CHO-OCT3) (B), and OCT3-mediated uptake in CHO cells (C) or by EGI-1 cells transfected with empty lentiviral vectors (EGI-1-Mock) (D), OCT3-expressing EGI-1 (EGI-1-OCT3) (E) and OCT3-mediated uptake in EGI-1 cells (F) in the absence (Control) or the presence of 10 μM quinine, regorafenib, lenvatinib, cabozantinib or sorafenib. Data are as means ± SD of 9 wells from three separate experiments. *, $p < 0.05$ on comparing with Control by the Bonferroni method of multiple-range testing. AUF, arbitrary units of fluorescence.

inter-cohort variability, the following features were observed: i) The known down-regulation of *SLC22A1* in HCC was confirmed in most datasets included in HCCDB (Fig. 3A-3B); ii) In most cohorts analyzed in the HCCDB, *SLC22A3* expression levels were moderately lower than that of *SLC22A1* (Fig. 3A-3C); iii) Interestingly, in contrast to *SLC22A1*, *SLC22A3* expression was basically preserved in HCC compared to adjacent non-tumor liver tissue (Fig. 3C-3D).

After confirming the ability of OCT3 to mediate sorafenib uptake and the preserved expression of this transporter in HCC, we set out to evaluate the relevance of OCT3 expression in HCC in predicting patient response to anti-tumor treatment with sorafenib in the TRANSFER cohort [9].

3.3. Patients' characteristics

Tissue samples included in this work were obtained from Caucasian patients. The samples were collected from surgically resected tumors or the remaining material of needle biopsies. The epidemiological and clinical characteristics of patients are shown in Table 1. The mean age of patients was 67 years, with a broad range of ages and a vast majority of men. Most had cirrhosis due to alcohol or viral (HVB or HCV) infections as underlying diseases. Two-thirds of the patients had a Child-Pugh class A score, 18% Child-Pugh class B score, and the rest could not be calculated due to unavailable laboratory values. Levels of serum transaminases and total bilirubin were elevated in some patients, while INR values were in the normal range in most of them.

3.4. Subcellular detection of OCT3 in HCC samples

Immunofluorescence study of OCT3 in healthy human liver cryosections with parallel labeling of the canalicular transporter MRP2 confirmed the presence of OCT3 in the basolateral membrane of hepatocytes (Fig. 4A). The primary antibody used in immunofluorescence analysis also worked in immunohistochemistry on FFPE tissue sections (Fig. 4B). Marked staining at the basolateral membrane of healthy hepatocytes was detected together with a slight intracellular signal. Next, immunohistochemical staining was performed on FFPE sections of HCC samples (Fig. 4C-4F). Considerable interindividual variability was seen. For tumor classification, the intensity of OCT3 protein labeling was scored as absent, weak, moderate, and strong, and, in addition, the presence or absence of the protein in the plasma membrane was taken into account. Fig. 4C-4F show representative images of each of the staining groups. It should be noted that in some cases, the heterogeneity of staining was observed in different areas of the same tumor (Fig. 4D). In these cases, the tumors were classified according to the expression in the predominant area. The fact that in some positive tumors the stromal cells lacked the specific staining (Fig. 4E) makes the labeling results more reliable.

Around 56% of assayed tumors showed positive OCT3 labeling with different intensity, from weak to strong (Fig. 5A), but in only 26% of HCC, this staining was localized at the plasma membrane (Fig. 5B). Therefore, based on this assay, it was estimated that in 74% of analyzed tumors, OCT3 was not expected to be involved in drug uptake by cancer cells.

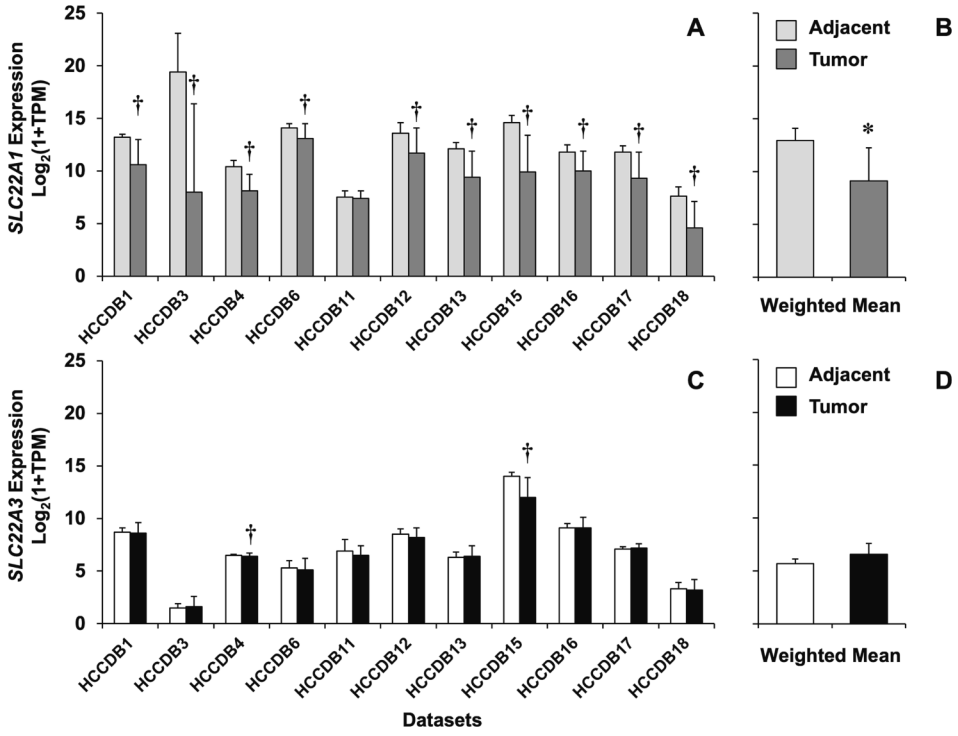

Fig. 3. Relative *SLC22A1* (A, B) and *SLC22A3* (C, D) expression levels obtained from different databases encompassed in the Integrative Molecular Database of hepatocellular carcinoma (HCC) (HCCDB). Mean probe values (log2 intensity) ± SD and p value are represented for each microarray dataset (A, C) or as a whole (weighted mean) (B, D) in samples collected from HCC and adjacent non-tumor tissue. Adapted from data available at http://lifeome.net/database/hccdb/home.html. The number of samples (Tumor/Adjacent) per database was as follows: HCCDB1: 100/97; HCCDB3: 268/243; HCCDB4: 240/193; HCCDB6: 225/220; HCCDB11: 88/48; HCCDB12: 81/80; HCCDB13: 228/168; HCCDB15 (TCGA): 351/49; HCCDB16: 60/60; HCCDB17: 115/52; HCCDB18: 212/177. Genes that were significantly differentially expressed (†, $p < 0.001$) in at least half of the analyzed datasets were identified as consistently differentially expressed (*) [19].

Table 1
Cohort's characteristics.

Number of patients	39
Age, mean (range)	67.4 (49–87)
Male gender, n (%)	35 (89.7)
Underlying disease, n (%)	
Cryptogenic cirrhosis	5 (12.8)
Hepatitis B virus	4 (10.3)
Hepatitis C virus	11 (28.2)
Alcoholic	11 (28.2)
Hemochromatosis	4 (5.1)
NAFLD	3 (7.7)
NA	3 (7.7)
Child-Pugh status, n (%)	
Child-Pugh A	26 (66.6)
Child-Pugh B	7 (18.0)
NA	6 (15.4)
Biochemistry (mean ± SD)	
ALT (IU/l)	51.3 ± 33.5
AST (IU/l)	63.8 ± 35.8
Total bilirubin (mg/dl)	4.3 ± 10.3
INR	1.1 ± 0.2

ALT, alanine aminotransferase; AST, aspartate aminotransferase; INR, international normalized ratio; NA, not available; NAFLD, non-alcoholic fatty liver disease.

3.5. Relationship between tumor OCT3 expression and patient survival

The relationship between OCT3 expression in HCC and OS from initiating systemic treatment with sorafenib is depicted as a waterfall plot in Fig. 6. Seven patients with expression of OCT3 at the plasma membrane were among the top ten survivors in this study, and five of them also presented OCT1 staining at the plasma membrane. Among the top ten survivors, one patient presented OCT1 but no OCT3 staining at the plasma membrane. No relationship between radiological response (partial response/stable disease *vs.* progression disease) or BCLC stage (A/B stage *vs.* C stage) was found ($p > 0.05$ by the Fisher's exact test).

In our previous study, we found that OS was higher ($p < 0.001$) in patients with HCC whose tumors had OCT1 expression in the plasma membrane than in those with tumors without this characteristic, including those whose staining was intracellularly localized [9]. A similar analysis was performed to determine whether the presence of OCT3 at the plasma membrane could contribute to improving the OS of these patients. Higher OS was found in patients whose tumor cells expressed one of the organic cation transporters (OCT1 alone or OCT3 alone) at the plasma membrane compared with those with tumor cells lacking any of these proteins in their plasma membrane (Fig. 7A). Furthermore, the log-rank test calculated considering the presence of any of both carriers (OCT3 or OCT1) in the plasma membrane of tumor cells compared with the absence of both transporters, resulted in better statistical significance ($p = 0.00017$) than considering only one of them separately compared with the absence of each of them ($p = 0.00040$ and $p = 0.00036$, for OCT1 or OCT3 alone in the plasma membrane *versus* the absence of each of them, respectively), i.e., the presence of either in HCC tumors has a similar ability to predict a better response to sorafenib treatment (Fig. 7B).

4. Discussion

Sorafenib was the first drug able to induce improved OS in patients with advanced HCC [20] and is still considered the first option for patients requiring systemic therapy who cannot benefit from therapy based on immunomodulators, such as atezolizumab plus bevacizumab or durvalumab plus tremelimumab, due to variceal bleeding risk, or because they have vascular disorders and arterial hypertension or severe autoimmune disorders [21]. Despite the significant role of sorafenib in

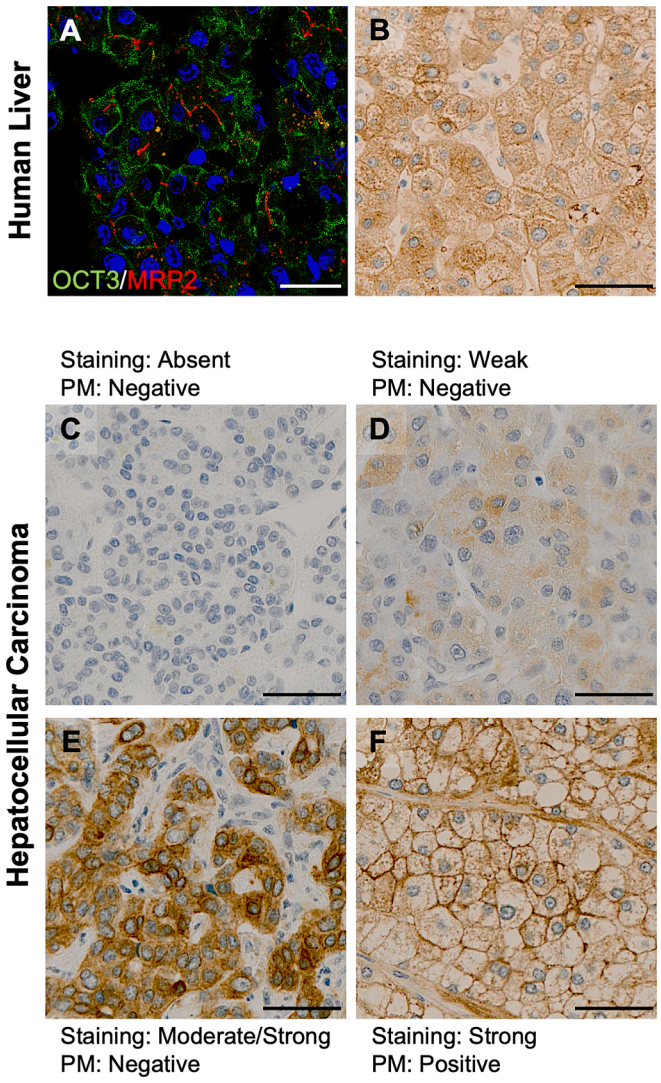

Fig. 4. Immunolocalization of OCT3 in healthy liver and hepatocellular carcinomas (HCCs). Immunolocalization of OCT3 (green), and MRP2 (red) in control human liver by immunofluorescence. Cell nuclei were stained with Dapi (A). Localization of OCT3 in hepatocytes in control human liver by immunohistochemistry and hematoxylin counterstaining (B). Representative images of OCT3 staining by immunohistochemistry and hematoxylin counterstaining showing the criteria used to classify HCCs according to the degree of intensity of OCT3 as absent (C), weak (D), moderate/strong (E, F) and according to the absence (C-E) or the presence (F) of staining at the plasma membrane of tumor cells. Bars: 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the treatment of advanced HCC, its benefit is moderate, the response rate is low [20], and adverse events that are associated with better response and lead to dose reduction are frequent [22]. Although new TKIs have been recently incorporated to the list of drugs approved to treat HCC, none of these compounds has demonstrated a markedly higher beneficial effect than sorafenib [23]. An additional question that hampers the use of sorafenib and other TKIs in the management of HCC patients is that, up to date, there is no reliable biomarker to predict the lack of response to treatment with these drugs.

Sorafenib is taken up by OCT1, and hence the impaired expression or activity of this transporter has been associated with a reduction in the beneficial effect of this drug [5]. Although the use of changes in *SLC22A1* mRNA to predict an unfavorable response to sorafenib treatment of HCC patients has been proposed [6], later studies have shown that the detection of OCT1 protein by IHC in the plasma membrane of tumor cells is more accurate in predicting the response to sorafenib [9]. Interestingly, in that study, a reduced group of patients without detectable OCT1 in the plasma membrane of tumor cells still had long OS. The question arose then as to whether another transporter could be mediating the access of sorafenib to its intracellular molecular targets. In this respect, OCT2 can be ruled out because this protein, mainly expressed in the kidney, is absent in hepatocytes. In contrast, OCT3, which is a polyspecific uptake transporter able to participate in the uptake of mainly cationic molecules has a wide tissue distribution, including liver cells [24]. Given that OCT1 and OCT3 can functionally substitute each other [11,12], a reasonable hypothesis was that OCT3 could be such a transporter contributing to the response of HCC to sorafenib. We decided to validate this hypothesis and extended the answer to the question as to whether other cationic TKIs that these patients may receive in different lines of treatment, i.e., lenvatinib, regorafenib, and cabozantinib, could also be taken up via OCT3.

Thus, using cells expressing OCT3, we demonstrated that this transporter could be involved in sorafenib uptake. Then we investigated by IHC the presence of this protein in a cohort of advanced HCC in which we had previously determined OCT1 expression [9]. Like what we had observed for OCT1, we found significant inter-tumor heterogeneity in OCT3 expression. Interestingly, although most tumors with OCT1 expression also expressed OCT3, which may contribute to better drug uptake and effect, the absence of OCT1 expression in HCC was not always accompanied by a lack of OCT3 expression. Our results agree with those described by others [15], who found that OCT1 but not OCT3 levels were related to tumor characteristics in HCC, and they did not change in parallel. However, high methylation levels of the *SLC22A3* promoter have been described in other solid tumors. Thus, genetic polymorphisms affecting *SLC22A3* promoter may be associated with OCT3 downregulation in HCC as happens in prostate cancer [25]. The presence of OCT3 at the plasma membrane suggests its functionality, although this feature is not guaranteed because >20 OCT3 variants with different functionality have been described regardless of their correct subcellular localization [26].

Despite no relationship between the presence of OCT3 in the membrane of tumor cells and clinical characteristics, such as tumor stage, radiological response, or the treatment before sorafenib administration was found (Fig. 6), there was a significant increase in OS in patients with tumors expressing presumably functional OCT3 (Fig. 7). Moreover, if we considered the overall OCT function, i.e., the presence of either OCT3 or OCT1 at the plasma membrane, the analysis of both of them by immunohistochemistry adds some advantages: i) some patients lacking OCT1 in the plasma membrane but expressing OCT3 could also be good candidates to receive sorafenib, ii) OCT1 is not able to transport regorafenib [10], but our results suggest that OCT3 could transport this drug, and other TKIs used in clinic, administered in patients who do not respond to sorafenib.

This work supports the previously proposed concept that plasma membrane transporters may be helpful as biomarkers for predicting response to antitumor treatment in liver cancer [27]. In this particular case, detecting OCT1 or OCT3 could be a good prognostic factor. However, it should be highlighted that since the lack of response to pharmacotherapy can be accounted for by a large variety of additive and synergic mechanisms of chemoresistance (MOCs), the presence of these transporters in the plasma membrane of cancer cells could predict drug uptake, but not the success of the treatment. Thus, other MOCs can influence the pharmacological effect of sorafenib, such as the overexpression of export pumps able to reduce the intracellular levels of the drug; changes in the activity in metabolizing enzymes enhancing sorafenib inactivation; or alterations in its intracellular mechanism of action, including changes in its targets or activation of alternative signaling pathways, i.e., in cancer cells with constitutively blocked RAF/MEK/ERK pathway [27]. For instance, breast cancer resistance protein (BCRP, *ABCG2*) has been proposed as a marker of response to sorafenib in HCC, as upregulation of BCRP in tumors has been correlated with

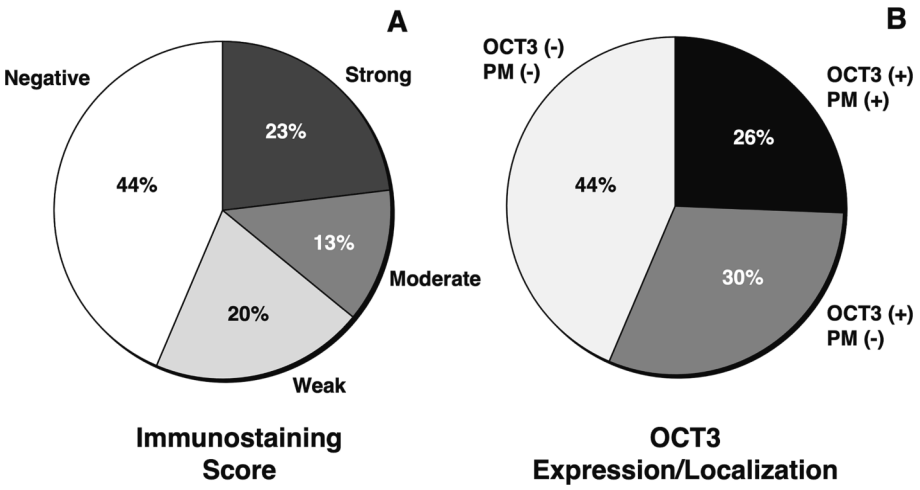

Fig. 5. Classification of patients with hepatocellular carcinoma (HCC) based only on the degree of OCT3 staining with anti-OCT3 antibody (A), or also considering the presence of the staining at the plasma membrane (PM) of HCC cells (B).

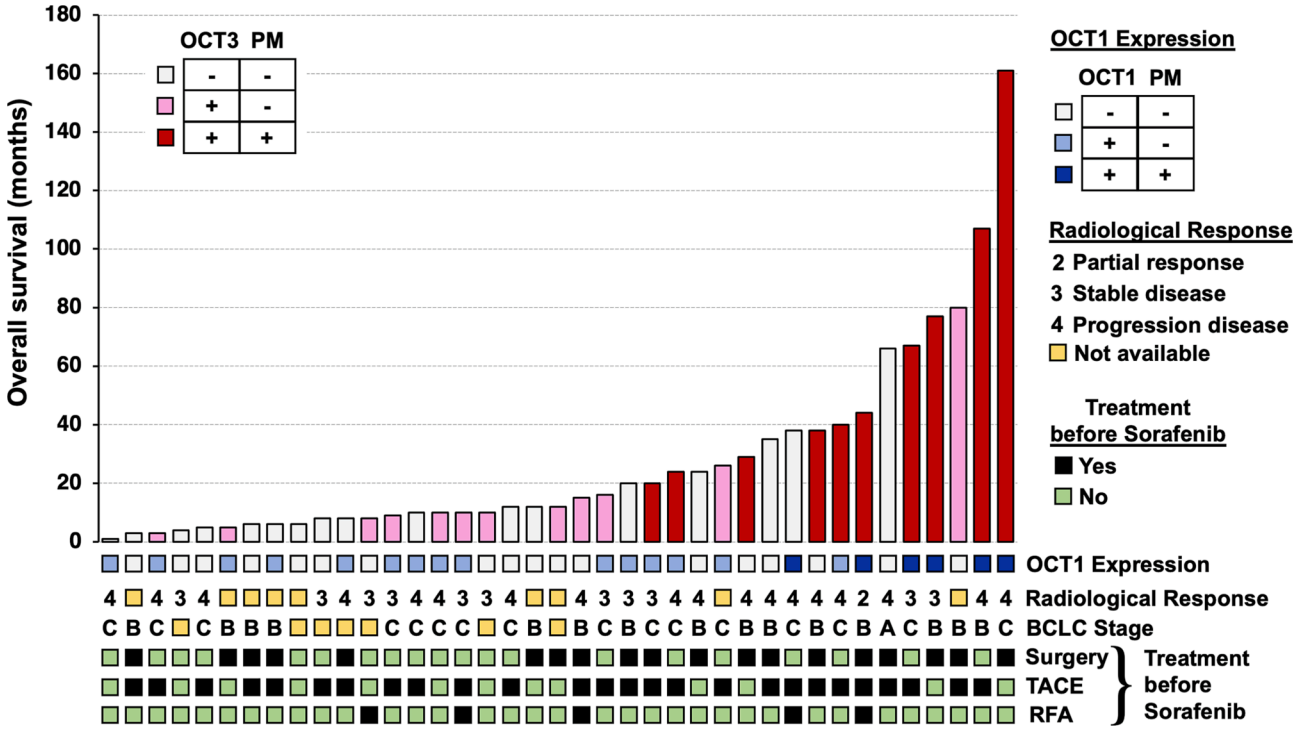

Fig. 6. Waterfall plot of the clinical response to sorafenib treatment as determined by overall survival after starting systemic pharmacological treatment with sorafenib. Patients were classified based on the intensity of OCT3 staining and the presence of the staining at the plasma membrane (PM) of tumor cells. OCT1 staining, radiological response, Barcelona Clinic Liver Cancer (BCLC) staging system and sorafenib pretreatment are also indicated for each patient. RFA, radiofrequency ablation; TACE, transarterial chemoembolization.

reduced OS in patients with HCC [28]. Multidrug resistance-associated protein 2 (MRP2, *ABCC2*) is involved in the transport of sorafenib, regorafenib, cabozantinib, and probably lenvatinib. It has been suggested that the presence of genetic variants of *ABCC2* that lead to upregulation of MRP2 or increase its exporting activity may reduce the sensitivity of HCC to such TKIs [29]. The presence of genetic variants in genes encoding TKRs or in elements of survival pathways that lead to an overactivation of the signaling pathway has also been associated with a worse outcome of HCC patients treated with TKIs [23].

The role of OCT3 in the pharmacokinetics of anti-HCC drugs can be likely extended to other TKIs. Thus, the results obtained in a cell model suggest that cabozantinib, lenvatinib and to a lesser extent regorafenib, are also able to interact with OCT3. The exact impact on the biodistribution of these drugs and the relation of OCT1 and OCT3 expression with the outcome of patients treated with them remain to be elucidated. Although a tissue sample is the preferred option, the identification of biomarkers in liquid biopsy samples would additionally overcome the challenge of limited tissue availability and allow monitoring the dynamic changes in MOCs occurring in tumor cells during the follow-up of these patients [30].

In conclusion, information on the presence of OCT1/OCT3 in the plasma membrane of HCC cells can be helpful for clinicians to select the most appropriate treatment.

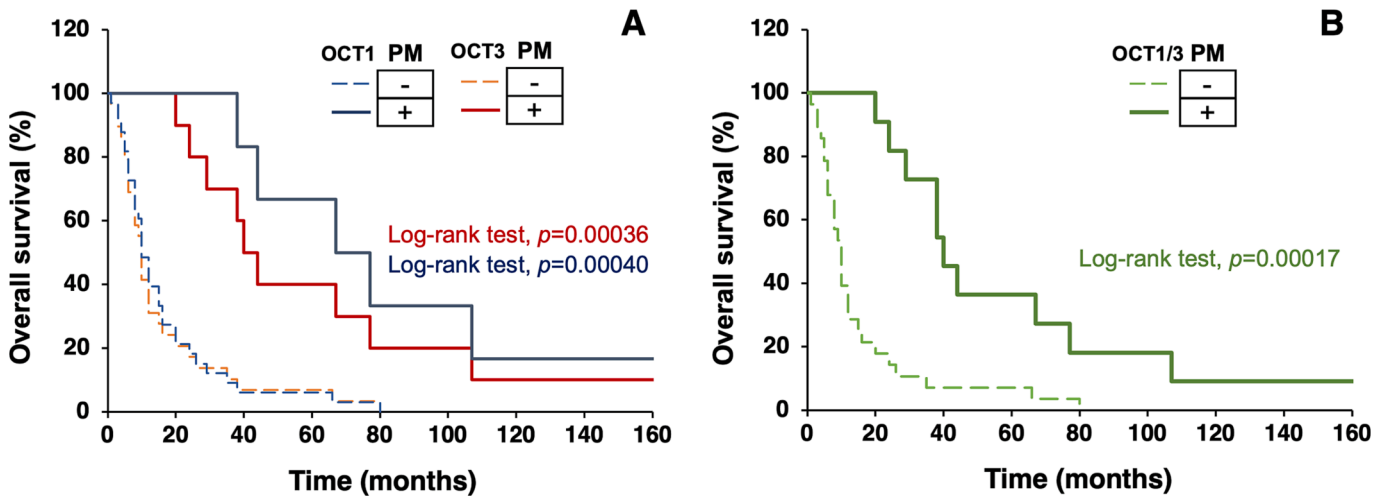

Fig. 7. Kaplan-Meier plots and log-rank statistics analyses of overall survival of patients with HCC after starting treatment with sorafenib. Patients were classified based on the absence or presence of the staining of OCT1 alone or OCT3 alone at the plasma membrane (PM) of tumor cells (A) or considering the absence of both transporters versus the presence of either OCT1 or OCT3 in the PM (B). Results were considered statistically significant when log-rank p value was < 0.05.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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