Correlation of Gene Expression of ATP-binding Cassette Protein and Tyrosine Kinase Signaling Pathway in Patients with Hepatocellular Carcinoma

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Abstract. *Background: Recent evidence suggests an involvement of the tyrosine kinase signaling pathway in the development of ATP-binding cassette (ABC) protein-mediated multidrug resistance in cancer. The aim of our study was to determine the relevance of kinase and multidrug-resistance protein expression in human hepatocellular carcinoma (HCC). Material and Methods: Paired tissue samples of HCC and corresponding peri-neoplastic tissue from 15 patients undergoing surgical resection were analyzed. The gene expression of ABC proteins and mitogen-activated protein kinase (MAPK) signaling cascade kinases was evaluated by real-time PCR and correlated with a series of clinicopathological parameters. In vitro effects of MAPK Kinase (MEK) inhibition were evaluated in HepG2 cells. Results: Overexpression of ABC proteins, tyrosine kinases, or both was detectable in 40%, 86% and 33% of HCC samples, respectively. ABCC1, -2 and -3-mRNA levels were significantly increased in 13%, 20% and 33% of the HCC samples compared to the corresponding peri-neoplastic tissue ($p \leq 0.05$). There was an association of ABCC1 and ABCC2 overexpression in HCC tissue ($p \leq 0.05$). EGFR, RAF, MEK, ERK and MAPK mRNA were overexpressed in 33%, 33%, 40%, 50% and 50%, respectively compared to the peri-neoplastic tissue ($p \leq 0.05$). The expression of ABCC1, ABCC2 and P-glycoprotein correlated statistically with the MEK gene expression. Patients with tyrosine kinase overexpression had significantly higher angioinvasion ($p \leq 0.05$). RAF overexpression correlated statistically with increased tumor size ($p=0.052$). In vitro, MEK inhibition led to a reduced ABCC1 mRNA and protein expression. Conclusion: ABC proteins and tyrosine kinases are significantly overexpressed in HCC tissue. The multidrug-resistance phenotype is associated with the MEK expression in HCC. Inhibition of MEK might be a new therapeutic approach to restore chemosensitivity in patients with highly resistant tumors.*

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy worldwide and it accounts for more than one million deaths annually (1). Liver resection and transplantation remain the only potentially curative treatment options for patients in an early stage of disease (2). However, recurrent disease occurs in up to 85% of patients (3). Those patients with advanced disease have a generally poor prognosis and the treatment options are mainly palliative. In recent years, the multikinase inhibitor sorafenib has become the current standard palliative firstline therapy (4). However, despite a significant prolongation of the time to progression being demonstrated in phase II-III trials, the clinical benefit is limited and the reported partial response rate is about 10% (5, 6). Conventional systemic chemotherapy has only minor effectiveness in HCC patients; none of the reported clinical trials showed a significant improvement in long-term survival (7).

HCC has a very complex molecular pathogenesis and shows high intrinsic drug resistance (2, 8). New approaches to overcome this resistance and offer patients tailored treatment strategies are urgently required. Beside alterations in the cell cycle and increased repair of DNA damage, the overexpression of ATP-binding cassette (ABC) proteins plays a pivotal role in the development of multi-drug resistance (MDR) in HCC (9). An increased expression and function of transmembrane drug efflux pumps has been described in HCC previously and is associated with poor clinical prognosis and biological aggressiveness (10-13). The regulatory mechanism of gene expression of ABC proteins has not been fully elucidated yet. There is increasing evidence that the mitogen-activated protein kinase (MAPK) pathway might be involved in the

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development and regulation of MDR in various tumor entities (14-16). Previously, our group showed an involvement of the epidermal growth factor receptor (EGFR) activated RAF/MAPK/MEK/ERK tyrosine kinase pathway in the regulation of MDR in HCC *in vitro* (17, 18). We demonstrated that cytostatic treatment increased the expression of tyrosine kinases and induced the phosphorylation of extracellular signal-regulated kinase (ERK). Furthermore, activation of the tyrosine kinase pathway by EGF induced MDR by up-regulating the ABC protein expression and enhanced the survival of resistant HCC cells. In contrast, EGFR inhibition restored chemosensitivity (17).

The aim of the present study was to validate the previous *in vitro* findings in human HCC tissue samples and to confirm a potential correlation of ABC protein and tyrosine kinase gene expression in HEPG2 cells.

Materials and Methods

Tissue samples. Fifteen paired tissue samples of histologically proven HCC and corresponding peri-neoplastic tissue directly obtained from the tumor margin of patients undergoing liver resection at the Department of General and Transplantation Surgery, Ruprecht Karls University Heidelberg between 2007 and 2009 were analyzed. The clinical characteristics of the patients are summarized in Table I. None of the patients received systemic or local therapy before liver resection. Informed consent was obtained from every patient and the study protocol conforms to the ethical guidelines of the Declaration of Helsinki (19).

Cells and chemotherapeutic treatment. The human HCC cell line HepG2 (Toni Lindl GmbH, Munich, Germany) was used for *in vitro* experiments, cultured in RPMI-1640/DMEM containing 10% fetal bovine serum (FBS) in 5% CO_2 at 37˚C. Gemcitabine (Lilly, Indianapolis, IN, USA), doxorubicin (Sandoz Pharmaceuticals GmbH, Holzkirchen, Germany) and U0126 (Cell Signaling, Beverly, MA, USA) were prepared according to the manufacturers' instructions. Cells were treated either with gemcitabine at 114 μg/ml, or doxorubicin at 0.6 μg/ml, or U0126 at 10 μM or a combination of chemotherapy and U0126 twice weekly.

Real-time-PCR. The quality of the tissue specimens was evaluated by a pathologist before RNA isolation. Total RNA was isolated from HCC or peri-neoplastic tissue and transcripted to cDNA according to the manufacturers' instructions (RNeasy Mini Kit, Qiagen, Hilden, Germany; Transcriptor First Strand cDNA Synthesis Kit, Roche Diagnostics, Basel, Switzerland). Semi-quantitative RT-PCR analysis was performed using Power SYBR Green as fluorescent probe and the StepOne RT-PCR System (Applied Biosystems, Foster City, CA, USA). Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as endogenous control. Commercially available primers for *GAPDH, ABCC1, ABCC2, ABCC3, PGP* and *EGFR* were used (Qiagen, Hilden, Germany). Primers for *RAF1*, *MEK, ERK* and *MAPK14* were designed using NCBI Primer-Blast software and produced by Invitrogen, Carlsbad, USA (Table II). In brief, after 10 minutes of denaturation at 95˚C, RT-PCR was carried out for 40 cycles at 95˚C for 15 s and extension at 60˚C for 60 s. The fluorescent signal was measured at the end of the annealing phase of each cycle. mRNA quantification was recorded and analyzed with the 2(-delta delta C(T)) method using the StepOne RT-PCR System (Applied Biosystems) (18). All samples were measured in triplicates. Two wells were used to monitor contamination in every run. The efficiency of primers was evaluated by serial dilution of cDNA and an efficiency of ≥85% was reached.

Table I. *Clinicopathological characteristics of HCC patients undergoing liver resection.*

Variable		
Gender, n (%)		
Male	11	(73%)
Female	4	(27%)
Median age, years (IQR)	68	(54-75)
Cirrhosis, n (%)		
Yes	8	(53%)
No	7	(47%)
Etiology of cirrhosis; n (%)		
Viral	3	(38%)
Alcoholic	1	(12%)
Combination	1	(12%)
Cryptogenic	3	(38%)
Child-Pugh score; n (%)		
A	7	(88%)
B	1	(12%)
C	-	
Mean AFP, ng/ml (SD)	946	(1248)
Median tumor size, cm (IQR)	5.5	(3.5-7.2)
Median number of nodules, (IQR)	1	(1-3)
Grading, n (%)		
G1	1	(6%)
G2	10	(67%)
G3	4	(27%)
Angioinvasion, n (%)		
Yes	9	(60%)
No	6	(40%)
UICC stage, n (%)		
I	3	(20%)
II	8	(54%)
IIIA	3	(20%)
IIIB	1	(6%)

AFP: Alpha-fetoprotein; SD: standard deviation; IQR: interquantile range.

Statistical analysis. SAS software (release 9.1; SAS Institute, Inc, Cary, NC, USA) was used for statistical analysis. Relationships between ordinal parameters were investigated using χ^2 test or Fisher's exact test if the number of patients was small. The correlation between mRNA expression in HCC tissue was assessed by Pearson correlation coefficient. Two-sided *p*-values were always computed and a difference of $p \leq 0.05$ was considered statistically significant.

Results

Gene expression of ABC proteins in HCC. RT-PCR analysis revealed an expression of *ABCC1, ABCC2, ABCC3* and *PGP* mRNA in HCC and corresponding peri-neoplastic tissue of each sample. HCC tissue mRNA expression levels higher than

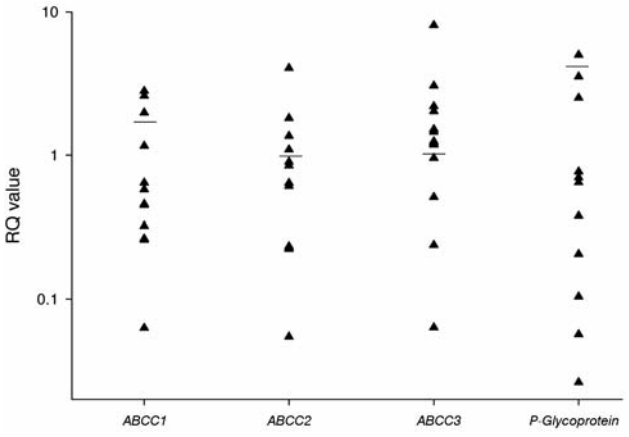

Figure 1. *mRNA Expression of ABC proteins in human HCC tissue after liver resection. Analysis of 15 tissue samples. Bars indicate mean+2SD mRNA level of peri-neoplastic tissue. RQ values are gene expressions relative to those of GAPDH.*

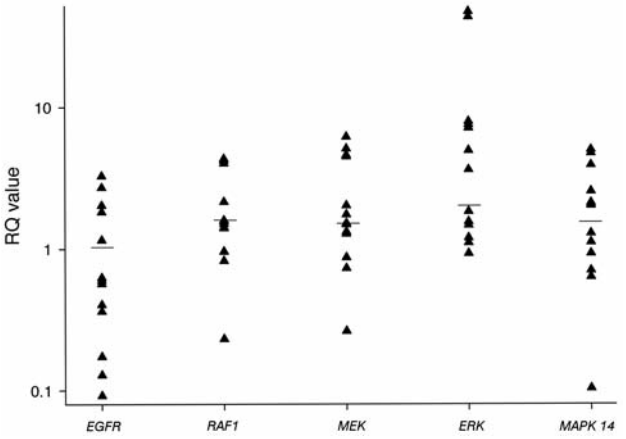

Figure 2. *Expression of tyrosine kinase mRNA in human HCC tissue after liver resection. Analysis of 15 tissue samples. Bars indicate mean+2SD mRNA level of peri-neoplastic tissue. RQ values are gene expressions relative to those of GAPDH.*

Table II. *Primer sequences.*

Protein	Forward primer	Reverse primer	Length (bp)
RAF1	5'-AGACTGCTCACAGGGCCTTA-3'	5'-CTGCAAATGGCTTCCTTCTC-3'	79
MEK	5'-GGTGTTTATTGGGCCTCAGA-3'	5'-ACCCGGAGCATCACAAATAG-3'	78
ERK	5'-GACAAGGGCTCAGAGGACTG-3'	5'-AGGACCAGGGGTCAAGAACT-3'	71
MAPK14	5'-TTGGTCAGTGGGATGCATAA-3'	5'-GGCTTGGCATCCTGTTAATG-3'	140

the mean+2SD mRNA level of peri-neoplastic tissue were classified as over expression. An overexpression of ABC proteins was detected in 40% (n=6/15) of HCCs compared to the surrounding peri-neoplastic tissue. *ABCC1, ABCC2* and *ABCC3* mRNA was overexpressed in 12% (n=2/15), 20% (n=2/15) and 33% (n=5/15) of HCC tissue compared to the corresponding peri-neoplastic tissue. The expression of *ABCC2* mRNA and *ABCC3* mRNA was 4-fold and 3-fold increased compared to the peri-neoplastic tissue ($p \leq 0.05$) (Figure 1). There was a significant association between ABCC1 and ABCC2 overexpression ($p=0.0461$, chi-square test).

Gene expression of tyrosine kinases in HCC. An overexpression of tyrosine kinase genes was detected in 80% (n=12/15) of HCC samples compared to the peri-neoplastic tissue. *EGFR, RAF1, MEK, ERK* and *MAPK* mRNA was overexpressed in 33% (n=5/15), 33% (n=5/15), 43% (n=6/15), 53% (n=8/15) and 53% (n=8/15), respectively, and increased 2-fold ($p \leq 0.05$), 3-fold ($p \leq 0.001$), 4-fold ($p \leq 0.001$), 4-fold ($p \leq 0.05$) and 2-fold ($p \leq 0.001$) compared to the corresponding peri-neoplastic tissue (Figure 2). There was a tendency towards an association of RAF-1 and MAPK overexpression ($p=0.064$, chi-square test).

Correlation between gene expression of ABC proteins and tyrosine kinase. An overexpression of both, ABC protein and tyrosine kinase genes was detectable in 33% (n=5/15) of patients. Of the patients with overexpression of ABC protein genes 83% had simultaneous tyrosine kinase overexpression. A significant statistical correlation of *ABCC1, ABCC2* and *PGP* overexpression with *MEK* overexpression was detectable ($p=0.0384$; $p \leq 0.001$ and $p \leq 0.001$), indicating that a higher gene expression of these ABC proteins in HCC is associated with a similar higher gene expression of *MEK* (Figure 3).

Correlation with clinicopathological parameters. Clinical (alpha-fetoprotein level, resection status and liver cirrhosis) and pathological factors (tumor size, number of nodules, grading, angioinvasion, UICC stage) were correlated with ABC protein and tyrosine kinase expression (Table III). HCCs with tyrosine kinase overexpression showed significantly more angioinvasion than tumors without tyrosine kinase overexpression ($p=0.017$). Furthermore, the *RAF1* mRNA expression had a tendency to be associated with an increased tumor size ($p=0.052$). During the follow-

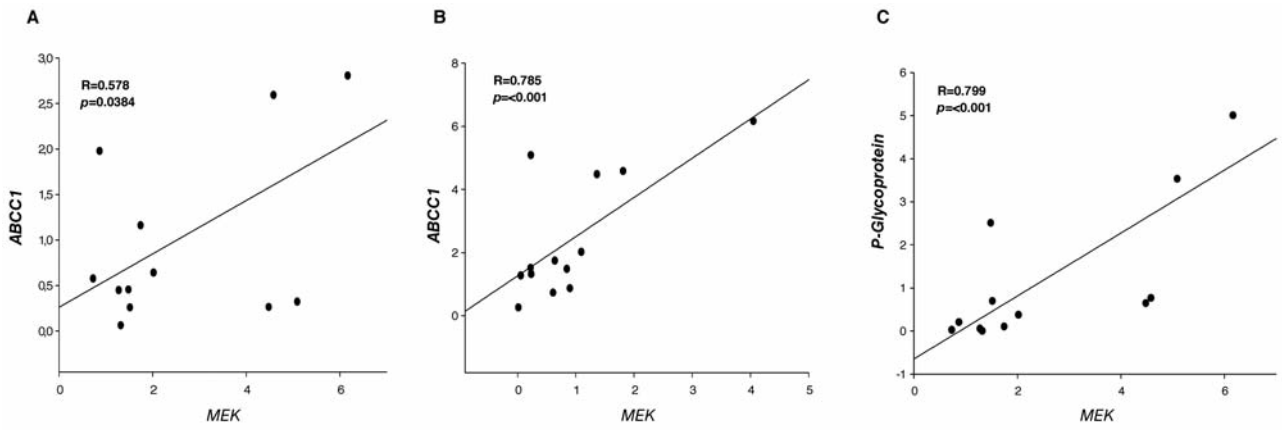

Figure 3. *Correlation analysis of mRNA expression of ABC protein and MEK in human HCC tissue. Analysis of 15 tissue samples.*

up period of 1-38 months (median 11 months) 8 of the 15 patients developed recurrent disease after liver resection. Recurrence occurred in 75% of patients with ABC protein and tyrosine kinase overexpression compared to 66% in patients with ABC protein overexpression only and 50% in patients with tyrosine kinase overexpression only. There were no significant differences in the time to progression, disease-free or overall survival.

Effects of MEK inhibition in vitro. Multidrug resistance was induced in HepG2 cells by conventional chemotherapy as described before (19). U0126 monotherapy reduced the expression of pERK protein significantly and there was a trend towards lower PGP and *ABCC1* mRNA expression (data not shown). To evaluate whether the addition of U0126 to conventional chemotherapy might restore chemosensitivity, the effects of gemcitabine plus U0126 treatment were analyzed. Simultaneous treatment of U0126 and conventional chemotherapy significantly reduced the *ABCC1* mRNA levels compared to monotherapy with gemcitabine ($p \leq 0.05$). Furthermore, it reduced the ABCC1 protein expression in resistant HepG2 cells (Figure 4A). Additionally, the relative viability of resistant HepG2 cells was reduced after combination therapy with U0126 (Figure 4B).

Discussion

The lack of effective treatment options, especially for patients with advanced disease, is one of the reasons for the high mortality and relatively low 5-year overall survival rates of 7% in patients with HCC (20). Alterations in important cellular signaling pathways have been linked previously with the treatment-resistant phenotype of HCC. Targeting these pathway alterations may help to restore chemosensitivity and offer new therapeutic approaches. The present study demonstrated that the MDR phenotype correlates with MEK expression in human HCC tissue and that MEK inhibition can reduce the ABCC1 expression *in vitro*.

In the last decade, improved knowledge of oncogenic processes during hepatocarcinogenesis has led to the identification of signaling pathways that regulate essential cell functions in cancer cells. The MAPK signaling cascade plays a key role in the signal transduction of cell differentiation, motility and proliferation after activation by extra- and intracellular ligands such as EGF and vascular endothelial growth factor (VEGF). These factors are significantly overexpressed in HCC (21). Furthermore, overexpression and enhanced activity of MAPK and ERK has been shown in HCCs in comparison to healthy liver tissue (22-24). Recently, an association of the MAPK pathway and MDR has been discussed. Cisplatin-induced ERK activation has been described in human cervical carcinoma cells (25). Our group has shown an involvement of the EGFR-activated RAF/MAPK/MEK/ERK tyrosine kinase pathway in the regulation of MDR in HCC *in vitro* (17;18). We demonstrated an EGF-induced up-regulation of ABC protein expression and enhanced survival of resistant HCC cells after tyrosine kinase activation for the first time. Furthermore, we showed potential therapeutic approaches to overcome this phenomenon by restoration of chemosensitivity through EGFR inhibition by gefitinib treatment or multikinase inhibition by sorafenib. However, the interaction between tyrosine kinases and the MDR mechanism remains unclear. It has been hypothesized that an increased phosphorylation of ABC proteins by activation of the EGFR/RAS/MAPK cascade or modulation of the MDR protein ATPase activity due to tyrosine kinase inhibition may play a role (26-28). Thus far, there have been few reports concerning a potential association of tyrosine kinases and the MDR phenotype in human cancer (14-16). Only one report by Guan *et al*. has demonstrated an increase in tyrosine kinase activity in resistant hepatoma cells (29). In the present

Table III. *Correlation between ATP-binding cassette (ABC) / tyrosinekinase (TK) overexpression and clinicopathological characteristics in patients with resected HCC.*

Variable n (%)	MRP overexpression		*P*-Value	TK overexpression		*P*-Value	MRP + TK overexpression		*P*-Value
	Yes	No		Yes	No		Yes	No	
	6 (40%)	9 (60%)		12 (80%)	3 (20%)		5 (33%)	10 (67%)	
AFP (ng/ml), mean±SE	784±555	634±356	0.982	948±496	942±578	0.991	1175±785	815±1161	0.927
Median tumor size, cm (IQR)	5.2 (2.9-4.7)	5.5 (2.6-3.8)	0.682	5.5 (2.4-3.5)	5.5 (3.3-5.6)	0.952	4.5 (3.0-5.7)	5.5 (5.0-8.3)	0.241
Median number of nodules, (IQR)	1 (2.3-3.5)	1 (0.8-1.1)	0.601	1 (1.2-1.8)	1 (1.3-2.2)	0.651	1 (1.0-2.5)	1 (1.0-3.0)	0.710
Tumor size									
<5 cm	2	2	0.199	3	1	0.223	2	2	0.223
5-10 cm	3	6		8	-		3	6	
>10 cm	1	1		1	1		-	2	
Tumor number									
1	4	5	0.199	7	2	0.223	3	6	0.199
2-3	2	3		2	-		-	3	
>3	0	1		3	-		2	1	
Grading									
G1	1	-	0.353	1	-	0.199	1	-	0.223
G2	3	7		8	1		3	7	
G3	2	2		3	2		1	3	
Resection status									
R0	6	6	0.287	9	3	0.223	5	7	0.223
R1	-	2		2	-		-	2	
R2	-	1		1	-		-	1	
Angioinvasion									
Yes	4	8	1.000	12	1	0.028	4	8	1.000
No	2	1		-	2		1	2	
Cirrhosis									
Yes	3	5	1.000	7	2	1.000	3	5	1.000
No	3	4		5	1		2	5	
UICC stage									
I	-	3	0.274	2	1	0.261	-	3	0.238
II	4	4		7	1		4	4	
IIIA	1	2		3	-		1	2	
IIIB	1	-		-	1		-	1	
Recurrence									
Yes	4	4	0.608	6	2	1.000	4	4	0.282
No	2	5		6	1		1	6	
Median TTR; days (IQR)	88 (64-113)	269 (164-455)	0.182	113 (79-432)	160 (88-318)	0.824	113 (109-151)	160 (67-458)	0.381

SE: Standard error; IQR: intequartile range; TTR: time to recurrence.

study, *ABCC1, ABCC2* and *ABCC3* mRNA was overexpressed in up to 33% of the analyzed HCC tissue samples compared to the corresponding peri-neoplastic tissue. This is in line with previous reports (11, 30). However, correlation with the tumor size or differentiation, as well as with the oncological outcome, was not detectable. One reason might be that patients included in this analysis were all untreated and had early HCC of small size and good differentiation to fulfill the resection criteria of our institution. Kato *et al*. analyzed patients undergoing liver resection who had in the majority of cases, multiple tumor nodules, and a tumor size over 4 cm was found in particular in those patients with high *PGP1* mRNA expression ratio. The multivariate analysis of their data revealed that the *PGP1* mRNA expression ratio is an independent predictive factor for overall survival after liver resection (12). Comparable to our data, 60% of patients with *PGP* mRNA overexpression showed recurrence in their analysis.

Human HCC was shown to exhibit a high expression of EGFR-associated downstream kinases (31-33). In the present

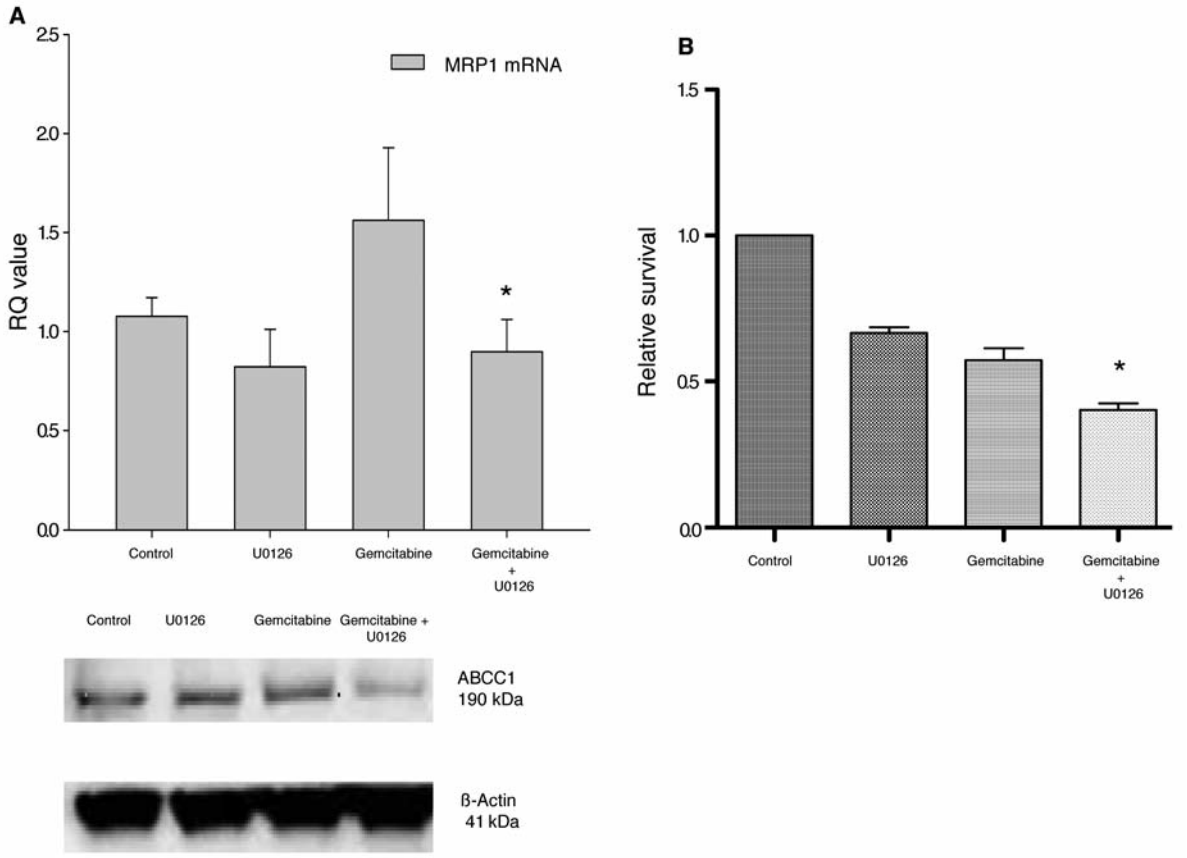

Figure 4. *A: ABCC1 mRNA and protein expression in HepG2 cells after treatment with U0126 10 μM, gemcitabine 114 μg/ml or gemcitabine 114 μg/ml plus U0126 10 μM. Columns are the average of three independent experiments, bars SD. *p≤0.05 compared to gemcitabine monotherapy. B: Relative viability of HepG2 cells after treatment with U0126 10 μM, gemcitabine 114 μg/ml or gemcitabine 114 μg/ml plus U0126 10 μM. Columns arte the average of three independent experiments, bars SD. *p≤0.05 compared to gemcitabine monotherapy. RQ values are gene expressions relative to those of GAPDH.*

study, overexpression of tyrosine kinases was detected in 80% of HCC samples compared to the peri-neoplastic tissue. The expression of tyrosine kinases were increased by up to 53%. Furthermore, patients with an overexpression of tyrosine kinases were found to have significantly more angioinvasion and a higher tumor size. In addition, our analysis demonstrated a close relationship between tyrosine kinase expression and mRNA levels for ABC proteins. Patients with overexpression of ABC proteins had simultaneous tyrosine kinase gene overexpression and recurrence occurred in 75% of those patients. A significant correlation of MDR protein expression with *MEK* gene overexpression was detected. MEK regulates the subcellular distribution of ERK, a downstream kinase involved in the nuclear phosphorylation of several transcription factors. ERK is mainly involved in the tumor cell proliferation of HCCs, participates in hepatocarcinogenesis during an early developmental stage, and has a central role in mediating invasion and metastasis (22, 24). Moreover, recent studies revealed that activation of the EGFR-pathway increased PGP expression in colorectal cancer cells and enhanced *ABCC1* gene expression in MCF-7 breast cancer cells (34, 35). At this point in time, we cannot sufficiently explain the interaction between tyrosine kinases and ABC proteins. Further studies are necessary to investigate the prognostic impact of combined tyrosine kinase and MDR. However, protein kinases have emerged as group of molecular targets which are inhibited by a new generation of chemotherapeutic agents. Based on the findings in human HCC tissue, we decided to analyze whether MEK may be a promising target and if its inhibition might influence MDR. Indeed, reduced ABCC1 expression was found after combined chemotherapy and U0126 treatment compared to chemotherapy alone. Additionally, the survival of resistant HCC cells was reduced after combination treatment with U0126.

In conclusion, the combination of a targeted drug treatment attacking singular pathway proteins with conventional chemotherapy might be a potential option to

restore chemosensitivity in patients with highly resistant tumors. However, further studies are needed to evaluate this approach in detail.

Acknowledgements

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