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Stimulus-induced expression of the *ABCG2* multidrug transporter in HepG2 hepatocarcinoma model cells involves the ERK1/2 cascade and alternative promoters

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ABSTRACT

The ATP-binding cassette G subfamily member ABCG2 protein is involved in drug resistance of various types of cancer including hepatocellular carcinoma (HCC). The transcriptional regulation of the ABCG2 gene was shown to depend on various transcription factors, and three alternative promoters were described. Here we aimed to decipher the role of hepatocyte growth factor (HGF) and the related kinase cascades on the expression of ABCG2 and the role of the different promoters in this process in the HepG2 human HCC cell line. We observed that HGF treatment increased the amount of ABCG2 on the cell surface in parallel with an increased ABCG2 transcription. ABCG2 mRNA expression was also increased by EGF, oxidative stress or activation of the aryl hydrocarbon receptor, while decreased by TGFb. Treatment with U0126, a specific inhibitor of the ERK1/2 cascade, prevented the HGF and the oxidative stress induced ABCG2 upregulation. We also show that the regulation of ABCG2 by various modulators involve specific alternative promoters. In conclusion, we demonstrate a unique role of the ERK1/2 cascade on ABCG2 modulation in HepG2, and the differential use of the alternative ABCG2 promoters in this cell line. This study reveals the molecular participants of ABCG2 overexpression as new potential treatment targets in HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent cancers and is a leading cause of cancer death. The incidence of HCC is high in East Asia and Africa while it is progressively increasing in the Western countries [1]. Various etiologies can lead to the development of this frequently fatal disease: virus infection (HBV or HCV), toxins or obesity. The role of several growth factors (GF) in this malignancy has been documented [2,3]. The constitutive activation of different tyrosine kinase pathways contributes to increased and maintained proliferation, invasion, vascularization and the inhibition of apoptosis, as well [2]. It is therefore not surprising that various mutations were detected among the members of the kinase cascades and that high levels of hepatocyte growth factor (HGF) was found to be correlated with poor prognosis and disease outcome [4]. Consequently, it is logical that the tyrosine kinase pathways are targets of HCC therapy. However, resistance to the treatments has already been observed.

The ABCG2 gene was originally cloned from drug-selected cancer cell lines [5–7] and later on was shown to be involved in the development of the multidrug resistance phenotype [8]. ABCG2 is expressed in various tissues, however, its exact cellular role and the nature of its transported physiological substrates remain elusive [9]. According to the current knowledge, the main function of this transporter is to extrude harmful substances from the cells to protect them from various noxae, and this role is supported by its high expression level in the liver, in tissue barriers and in stem

Abbreviations: ABCG2, ATP-binding cassette G subfamily member 2; AhR, aryl hydrocarbon receptor; BIMI, bisindolylmaleimide I; BNF, beta naphtoflavone; EGF, epidermal growth factor; GF, growth factors; HCC, hepatocellular carcinoma; HGF, hepatocyte growth factor; PMA, phorbol myristoyl acetate; *t*-BHQ, *tert*-butyl hydroquinone; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TGF, transforming growth factor.

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cells [10,11]. ABCG2 is suspected to play a role in the development of various cancers and recently it has been suggested to have a crucial role in HCC [12,13].

While the transport function of ABCG2 is well documented [14], fewer reports have been published on the transcriptional regulation of the gene, although some modulators of its transcription have already been described [15–18]. In this study we investigated the potential role of growth factors on the regulation of ABCG2 gene expression in the hepatocellular carcinoma cell line HepG2, a cell line frequently used as a model of HCC. We observed an increase in ABCG2 transcription after treatment with HGF and oxidative stress. Further analysis identified the ERK1/2 cascade as the main actor of this induction. Analysis of ABCG2 mRNA variants after treatment with different modulators of ABCG2 expression showed that this regulation is due to a complex mechanism involving the alternative promoters A and B. Altogether, our results provide evidence for a pivotal role of the ERK1/2 cascade in ABCG2 modulation in the hepatocarcinoma cell line HepG2, and permit a deeper understanding of ABCG2 transcriptional regulation.

2. Materials and methods

2.1. Cell culture

HepG2 human hepatoma cell line was obtained from ATCC and cultured according to the manufacturer's instructions. For drug treatments, cells were cultured in serum free medium, and treated for 24 h with the indicated compound. For co-treatment the different inhibitors were added 1 h before addition of the activators as described previously [19]. Chemicals obtained from Sigma–Aldrich were used at the following concentrations: human recombinant HGF 40 ng/ml, epidermal growth factor (EGF) 100 ng/ml, TGFbeta 5 and 10 ng/ml, phorbol myristate acetate (PMA) 100 nM, menadione 5 μM, *tert*-butyl hydroquinone (*t*-BHQ) 75 μM. From Calbiochem: U0126 2 μM, bisindolylmaleimide I (BIMI) 500 nM, LY294002 20 μM.

2.2. Flow cytometry analysis

ABCG2 cell surface expression was measured by flow cytometry (BD Biosciences FACSCalibur) using the conformation sensitive 5D3 primary antibody (BD Biosciences, CDw338, catalog number 552823) and a phycoerythrin-labeled anti-mouse secondary antibody (Molecular Probes, catalog number P-21149). The 5D3 signal was maximized by the addition of 5 μM of Ko143 (Tocris, catalog number 3241), a specific ABCG2 inhibitor [20].

2.3. Quantitative PCR (qPCR)

mRNA expression levels of ABCG2 were measured after extraction of total RNA using TRI REAGENT (Molecular Research Center, Inc.). Half microgram of RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

2.4. SYBR Green

LightCycler FastStart DNA Master SYBR Green I (Roche) was used in an LC480 quantitative PCR machine (Roche) in a 10 μl reaction volume. Primer sequences are listed in Table 1; ABL was used as an internal control, and the relative concentrations were calculated using calibration curves as described previously [19,21].

2.5. TaqMan

To measure the levels of alternative ABCG2 mRNA transcripts differing in their 5′-UTR, variant-specific TaqMan® assays with 5′-FAM and 3′-MGB labels were designed and tested for their specificity and their efficiency. All assays were found to be variant specific and had efficiency values of $\geqslant$90% (not shown). Primer sequences are listed in Table 1. To compare the expression levels with the total ABCG2 mRNA pool, a pre-designed TaqMan® assay was purchased from Applied Biosystems targeting the 3′ region of the mRNA present in all variants ("total ABCG2", assay ID: Hs01053790 m1); RNA polymerase IIA was used as an endogenous control (assay ID: HS00172187 m1). All reactions were performed on the StepOnePlus™ platform with reagents suggested by the manufacturer (Applied Biosystems).

3. Results and discussion

3.1. HGF modulates ABCG2 protein expression level at the plasma membrane

In HCC, HGF receptor and the downstream ERK1/2 are highly active. First, we tested whether these pathways have any effect on ABCG2 protein expression in the HepG2 human hepatocellular carcinoma cells. After a 24 h treatment of the cells with HGF, ABCG2 protein level was measured by flow cytometry analysis (Fig. 1A). We observed a significant increase of ABCG2 protein expression on the cell surface. These data indicate that the activity of this growth factor pathway gives a double advantage to the cancerous cell: first by inducing its replication and second by increasing its potential resistance against chemotherapy, due to the increased amount of ABCG2 protein in the cell membrane.

3.2. Growth factors differentially modulate ABCG2 expression in HepG2 cells

In order to decipher the mechanisms of the observed increase in ABCG2 level, in the following experiments we aimed to test whether various growth factors (GF) with major role in hepatic metabolism and carcinogenesis (HGF, epidermal growth factor (EGF) and transforming growth factor Beta (TGFbeta)) have any effect on the expression of ABCG2 at the transcriptional level. HepG2 cells were treated with the GFs and the relative ABCG2 mRNA expression levels were subsequently analyzed by qRT-PCR.

We observed that HGF and EGF treatments increased, while TGFbeta decreased the expression of ABCG2 (Fig. 1B). These results indicate that, in contrast to HGF and EGF, TGFbeta does not confer

Table 1
Primers used in this study.

Gene	Forward 5′–3′	Reverse 5′–3′	Probe TaqMan 5′-3′
ABCG2	CCGCGACAGTTTCCAATGACCT	GCCGAAGAGCTGCTGAGAACTGTA	
ABL	GGGCTCATCACCACGCTCCA	CTGCCGGTTGCACTCCCTCA	
ABCG2 A variant	GTGACGGCGACCAAACC	ACATTACTGGAAGACATCTGGAGAGT	CTAGGTCAGACGAGAAAGA
ABCG2 B variant	CCTTTGGTTAAGACCGAGCTCTATT	TGACACTGGGATAAAAACTTCGACAT	AAGCTGAAAAGATAAAAACTCTCC
ABCG2 C-variant	GAGATTTGGGCTGCTTTGCTT	ACATTACTGGAAGACATCTGGAGAGT	CACATCATAACTGAGAAAGAT

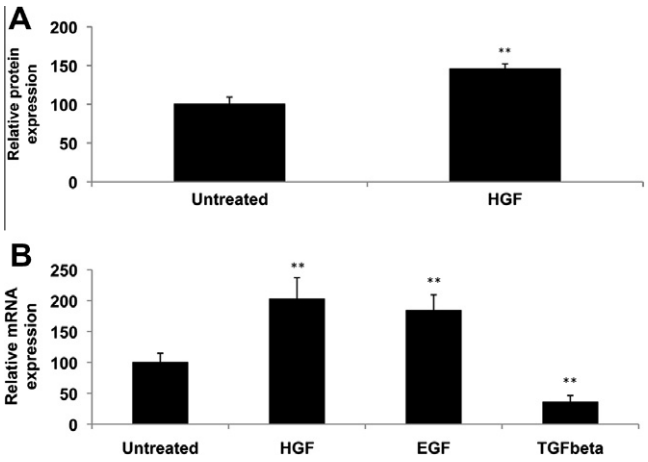

Fig. 1. (A) HGF treatment increases ABCG2 expression level in the plasma membrane. ABCG2 on the cell membrane was determined by flow cytometry using the 5D3 antibody directed against ABCG2 ($n = 3$). (B) Growth factors differentially modulate ABCG2 at the transcriptional level. The relative expression of ABCG2 was evaluated by qPCR ($n = 4$). $^{*}p < 0.05$; $^{**}p < 0.01$. Results are indicated in percentage relative to the control.

the advantage of developing cancer resistance via induction of ABCG2 to the cancerous cell. Other studies are consistent with our observations and support the potential role of HGF in the modulation of ABCG2 expression. Indeed, both in human primary hepatocytes and in hepatocarcinoma the activation of the HGF receptor (c-met) by HGF was shown to upregulate the expression of ABCG2 [22,23].

To further study the observed effects of the GFs, we next investigated the expression level changes of the alternative promoters of ABCG2 (Fig. 2A). Three alternative promoters were described for the ABCG2 gene in humans and mice. These promoters were linked to tissue-specific expression and drug resistance [24,25]. As a preliminary experiment, we tested the presence of the different variants in the HepG2 cell line. While the most distant C-variant remained undetectable both under control and treated conditions, we easily detected the A and B variants. We observed approximately twice as much B than A variant at the basal conditions (not shown). This finding suggests a potential inherent mechanism of fine-tuning of the ABCG2 expression level via the independent transcriptional regulation of the different variants.

When examining the effects of TGFbeta treatment, an approximately 50% decrease was observed, with an almost identical change in both mRNA variants and the global ABCG2 mRNA level. In contrast, both mRNA variants were induced by HGF (Fig. 2B). However, the A variant was somewhat more increased than the B variant, suggesting that the effect was at least partially transcriptional. The fact that both variants were induced, but to a different extent, also suggests that more than one mechanism is implicated in the regulation of the gene by HGF [10,24,25]. In order to understand these mechanisms we investigated the upregulation of ABCG2 expression by HGF in more detail.

3.3. PI-3k partially activates the expression of ABCG2 without any variant specificity

It is known that HGF can activate three major kinase cascades: PI-3k, PKC and ERK1/2 [2]. We systematically tested the implication of the different pathways in the regulation of ABCG2 by the use of specific activators and inhibitors. First, treatment with LY294002, a specific inhibitor of PI-3k, led to a partial prevention of the activation of the total ABCG2, more pronounced for the A variant and similar to total ABCG2 for the B variant, suggesting a partial involvement of PI-3k in the expression modulation of ABCG2 (Fig. 2C). This observation is in concordance with various studies where PI-3k was shown to modulate the expression of ABCG2 [26].

3.4. Phorbol ester activates the expression of ABCG2

We next performed co-treatment of HepG2 cells with HGF or PMA, a specific activator of PKC, and BIMI, a specific inhibitor of PKC. Both HGF and PMA treatments induced ABCG2 expression,

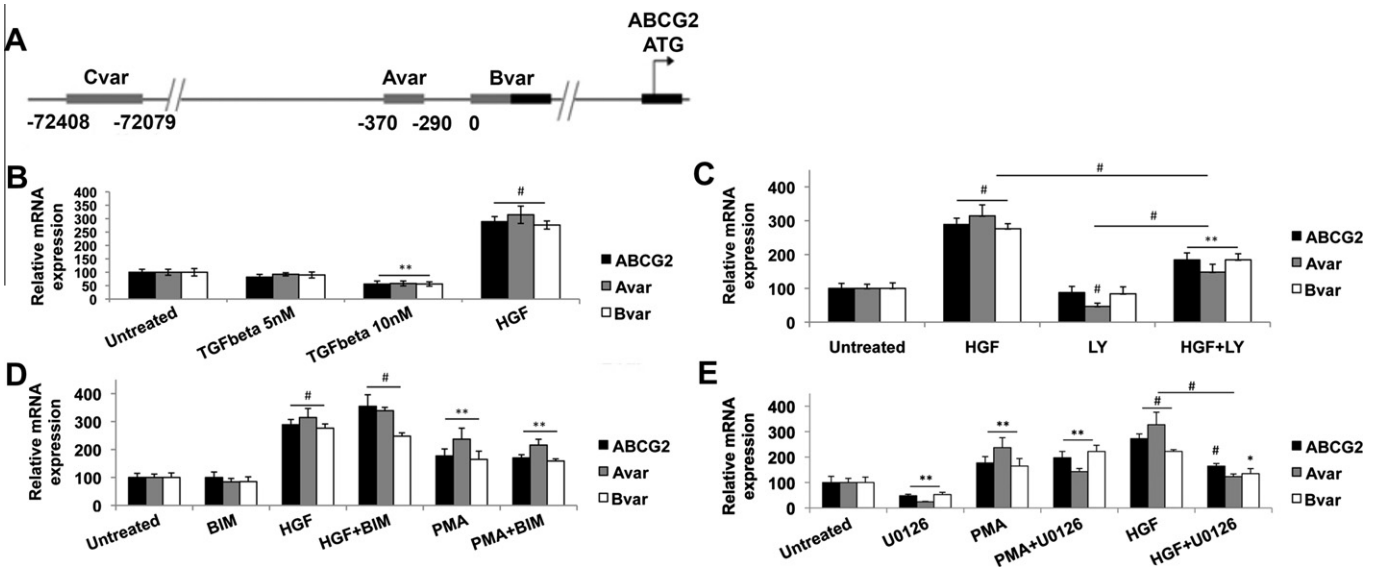

Fig. 2. The ERK1/2 cascade is mainly responsible for ABCG2 induction after HGF treatment. The relative expression of ABCG2 and its variants were evaluated by qPCR. (A) Schematic representation of the localization of human ABCG2 variants (numbers are distances in nucleotides from B variant transcriptional start). (B) Transcriptional modulation of ABCG2 variants by growth factors. Cells were treated with HGF or TGFbeta. (C) HGF induces ABCG2 expression partially *via* the PI-3k. Cells were treated with HGF or LY294002 (indicated as LY on the figure) or in combination. (D) PKC modulates ABCG2 expression. Cells were treated with HGF, PMA, BIMI or their combination. (E) HGF induces ABCG2 expression *via* the ERK1/2 kinases. Cells were treated with HGF, PMA, U0126 or in combination ($n = 4$). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{\#}p<0.001$. Results are indicated in percentage relative to the control.

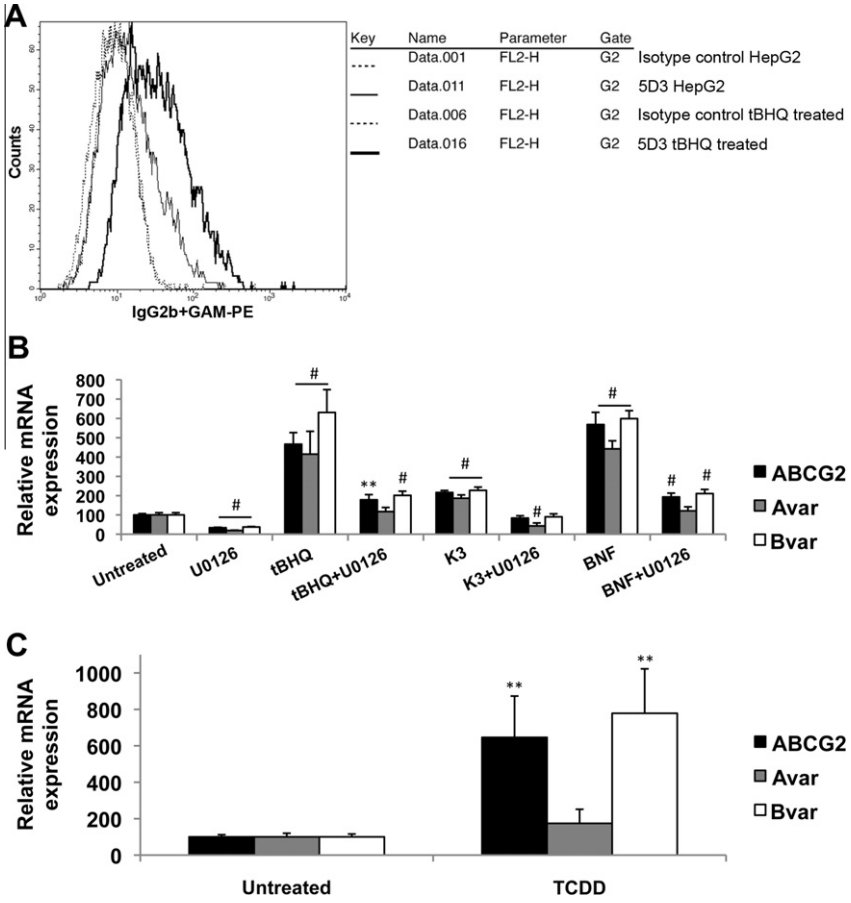

Fig. 3. Measurement of ABCG2 expression by flow cytometry and qPCR. (A) ABCG2 protein amount on the cell membrane was determined by FACS using 5D3 antibody directed against ABCG2 with or without *t*-BHQ treatment. (B) oxidative stress induction of ABCG2 variants is prevented by the specific inhibitor of the ERK1/2 cascade. Cells were treated with *t*-BHQ or Vitamin K3 (labeled K3 on the figure) or BNF three oxidative stress inducers or U0126 or in combination as determined by qPCR ($n = 4$). (C) ABCG2 modulation by AhR is specifically due to the modulation of the B variant as determined by qPCR ($n = 4$). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{\#}p < 0.001$. Results are indicated in percentage relative to the control.

and co-treatment with BIMI did not significantly prevent either PMA or HGF induction. We therefore hypothesized that neither classical nor novel PKC subfamily members are involved in ABCG2 activation but PMA might activate directly the MAP kinase pathway and increase the expression of HGF *via* this mechanism (Fig. 2D). We tested this hypothesis in the following experiments.

3.5. HGF activates the expression of ABCG2 via the ERK1/2 cascade

We performed treatment with U0126, a specific inhibitor of ERK1/2, to test the role of this kinase in ABCG2 regulation. Interestingly, treatment with U0126 alone led to an inhibition of more than 50% of the expression of both variants, and we observed that the A variant was much more sensitive to U0126 than the B variant, supporting the hypothesis of the distinct transcriptional modulation of the variants. We also observed that U0126 is able to completely prevent the induction of ABCG2 after HGF treatment (Fig. 2E), which clearly confirmed the involvement of the ERK1/2 cascade in ABCG2 transcriptional modulation. Co-treatment with U0126 partially prevented the PMA induction only on the A variant, confirming the involvement of the ERK1/2 cascade in ABCG2 modulation. However, the same treatment had much smaller effect on the B variant and on total ABCG2 mRNA levels. Although it is well documented that PKC can activate the ERK1/2 cascade, our data indicate that the induction of ABCG2 expression via PKC has an ERK1/2 independent component as well.

3.6. Oxidative stress induces ABCG2 in an ERK1/2 dependent manner and without specificity for any of the variants

It is well known that oxidative stress induces the ERK1/2 pathway and cancerous cells often have to cope with oxidative stress. Moreover, ABCG2 expression is induced following oxidative stress ([16,17] and Fig. 3A). Therefore we decided to investigate the possible involvement of ERK1/2 in the regulation of ABCG2 by oxidative stress response. We tested the effect of oxidative stress on the expression of the alternative variants of ABCG2. Treatment with three oxidative stress inductors, *t*-BHQ, Vitamin K3 [27] and beta naphtoflavone (BNF) [28], increased the expression of ABCG2, while co-treatment with U0126 almost completely prevented this induction. Notably, oxidative stress had a more pronounced effect on the B variant, as compared to the A variant, suggesting first that the effect is transcriptional, and also that the observed induction is not only ERK1/2 dependent as this cascade acts primarily on the A variant (Fig. 3B).

3.7. AhR agonist TCDD activates the expression of ABCG2 specifically via the B variant

Different inhibitors of the HGF, ERK1/2 pathway are used in chemotherapy. Since the inhibition of the pathway also inhibits ABCG2 expression, this approach has a double advantage. However, data from the literature suggest that these molecules might increase the expression of ABCG2 via an alternative mechanism:

the activation of aryl hydrocarbon receptor (AhR). It is noteworthy that AhR has recently been characterized as a modulator of ABCG2 expression and its DNA binding sites have been identified [15,29].

The results we obtained by BNF treatment led us also to investigate the potential effect of AhR on the expression of ABCG2 promoter variants as BNF has been shown to activate both Nrf2 (by inducing oxidative stress) and AhR. Treatment of HepG2 cells with TCDD, a specific activator of AhR, led to an important induction of endogenous ABCG2 (700%) (Fig. 3C). However, surprisingly the A variant did not exhibit any modulation after the treatment, suggesting that the ERK1/2 cascade is not involved in that modulation, despite the fact that TCDD has been shown to be able to activate ERK1/2 in some cases [30]. Finally, this also shows that the induction of ABCG2 expression by AhR is specific to the B promoter-variant in HepG2 cells although three AhR binding sites (one in the A variant and two between the A and the B variants) were reported to regulate ABCG2 expression in different cell types [15,29].

In conclusion, we have shown that HGF induces the expression of ABCG2 via the ERK1/2 cascade and we demonstrated that inhibition of this cascade in HepG2 cells causes the inhibition of ABCG2 expression at both transcriptional and protein expression level. We have also shown that the ABCG*2* variants are differentially modulated in HepG2 cells. This observation suggests a complex transcriptional regulation of ABCG2 pointing toward an eventual independent role of the different ABCG2 variants. Our data may be interpreted as a potential link between the invasiveness of the tumor and its ability to resist anti-cancer chemotherapy.

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